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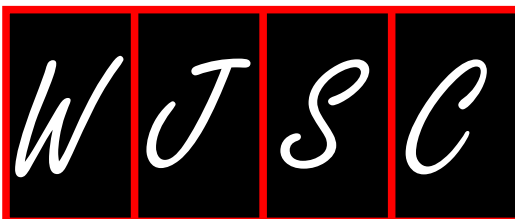
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Preventing aging with stem cell rejuvenation: Feasible or infeasible?

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Abstract

Characterized by dysfunction of tissues, organs, organ systems and the whole organism, aging results from

the reduced function of effective stem cell populations. Recent advances in aging research have demonstrated that old tissue stem cells can be rejuvenated for the purpose of maintaining the old-organ function by youthful re-calibration of the environment where stem cells reside. Biochemical cues regulating tissue stem cell function include molecular signaling pathways that interact between stem cells themselves and their niches. Historically, plasma fractions have been shown to contain factors capable of controlling age phenotypes; subsequently, signaling pathways involved in the aging process have been identified. Consequently, modulation of signaling pathways such as Notch/Delta, Wnt, transforming growth factor- β , JAK/STAT, mammalian target of rapamycin and p38 mitogen-activated protein kinase has demonstrated potential to rejuvenate stem cell function leading to organismic rejuvenation. Several synthetic agents and natural sources, such as phytochemicals and flavonoids, have been proposed to rejuvenate old stem cells by targeting these pathways. However, several concerns still remain to achieve effective organismic rejuvenation in clinical settings, such as possible carcinogenic actions; thus, further research is still required.

Key words: Aging; Stem cell; Niche; Rejuvenation; Signaling pathway

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Core tip: Functional loss of stem cells plays an important role in organismic aging processes. Recent advances in aging research have uncovered the molecular mechanisms of aging, specifically signaling pathways involved in interactions between stem cells and their environment, the so-called "stem cell niche". Investigating plasma fraction factors has revealed several key pathways involved in this process, including Notch/Delta, Wnt, transforming growth factor- β , JAK/STAT, mammalian target of rapamycin and p38 mitogen-activated protein

kinase signaling. Stem cell rejuvenation has the potential to lead organismic rejuvenation by modulating these pathways, hopefully by synthetic or natural agents such as phytochemicals and flavonoids.

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INTRODUCTION

Preventing pathological conditions caused by aging, including cancer, osteoporosis, sarcopenia, and cognitive disorders, is one of the most important issues for human health, especially in societies with large aging populations. Although aging, defined by functional decline of cells/organs or accumulation of cell/organ damage, is one of the most recognizable biological characteristics in all creatures, our understanding of mechanisms underlying the aging process remains incomplete. The primary cause of functional declines occurring along with aging is considered to be the exhaustion of stem cell functions in their corresponding tissues. Stem cell exhaustion is induced by several mechanisms, including accumulation of DNA damage and increased expression of cell cycle inhibitory factors, such as *p16* and *p21*^[1].

Meanwhile, aging at cellular, tissue, organ and organismic levels has been reversed by exposing tissues from old animals to a young environment. Recent studies have suggested that stem cell rejuvenation could reverse organismal aging phenotypes, and that this could be achieved by inhibiting fibroblast growth factor 2^[2], mammalian target of rapamycin (mTOR) complex 1^[3], guanosine triphosphatase and cell division control protein 42^[4]. Several additional experiments, such as cross-age transplantation and heterochronic parabiosis, have revealed that some factors in the young systemic milieu can rejuvenate declined thymus gland function, as well as neural and muscle stem cell functions, in samples derived from elderly donors^[5,6]. Furthermore, heterochronic parabiosis experiments have also shown strong inhibition of young tissue stem cells by the aged systemic milieu or old serum^[6].

Although cumulative cellular "intrinsic changes", such as DNA damage, oxidative damage, increased expression of cell cycle inhibitors and mitochondria dysfunction, have been considered likely culprits for the tissue decline observed with aging, cellular rejuvenation induced by young systemic milieu would have been impossible if "intrinsic changes" were the only cause of cellular aging. Therefore, these so-called "causes of aging" should be more properly regarded as effects of aging (*i.e.*, these processes are not causes, but rather consequences of aging), the result of cellular decisions

often defined by responses to "extrinsic stimuli".

Here some questions arise: If aging at the cellular level were reversed, would it lead to the rejuvenation of the animal at an organismic level? Would it result in prevention of aging and, eventually, life extension? In this editorial, the feasibility of stem cell rejuvenation will be discussed with specific focus on attenuation or reversal of tissue aging.

HISTORY OF REJUVENATION RESEARCH

Numerous studies have shown experimental rejuvenation at cellular, tissue, organ and organismic levels. The first experiment to investigate the possibility of animal rejuvenation, performed by McCay *et al*^[7] used the uncommon technique of "parabiosis", or surgically joining the circulatory systems of two animals. They observed old rats that had been sutured to young rats to establish heterochronic parabiosis appeared younger by visual appearance of tissues (mostly non-cellular cartilage). In 1972, Ludwig *et al*^[8] performed similar, but more quantitative experiments demonstrating life extension in older animals, who benefitted from sharing the blood supply of younger animals.

Cross-age transplantation studies have also indicated the rejuvenation potential of tissue and organs. The first cross-age transplantation study of muscle, conducted by Carlson *et al*^[5], showed that the mass and maximum force of old muscle grafted into young hosts were not significantly different from those of young muscle grafted into the same young hosts. Conversely, young muscle grafted into old hosts regenerated no better than old muscle grafted into the same old hosts. Hence, they concluded that chronological age alone is not a limiting factor for the intrinsic ability of muscle to regenerate. Further, poor regeneration of muscle in old animals is a function of the regenerative environment provided by the old host^[5]. A thymus transplantation study also showed that senescent, involute thymus glands became fully functional upon transplantation into young animals^[9].

Experiments undertaken by Lanza *et al*^[10] demonstrated that nuclei of senescent cells are repairable, as evidenced by the productivity of normal offspring from bovine ova containing nuclei transplanted from senescent cells.

In 2005, Conboy *et al*^[11] showed that stem cell tissues of older rats became phenotypically younger than age-matched controls when these animals were exposed to a young systemic environment. Further, differentiated cells can be reprogrammed to an embryonic-like state by transfer of nuclear contents into oocytes or by fusion with embryonic stem cells (ESCs). Next, Takahashi *et al*^[12] demonstrated induction of pluripotent stem cells (iPSCs) from mouse embryonic cells or adult fibroblasts by introducing four factors (Oct3/4, Sox2, c-Myc, and Klf4) under ESC culture conditions in 2006. Lapasset *et al*^[13] further demonstrated that iPSCs

derived from centenarians were rejuvenated such as to be indistinguishable from those derived from youthful cells.

At the organismic level, recent heterochronic parabiotic experiments pairing young and old rats resulted in increased neurogenesis and functional improvement of cognitive ability in the older parabiotic partner; whereas the younger partner exhibited decreased neurogenesis and cognitive abilities, consistent with that of an older rat^[14].

All of these studies raised the question as to whether cellular rejuvenation would have been possible if aging at the cellular level only resulted from accumulation of damage and/or toxic metabolic byproducts. The answer appears to be “no”, as aging seems to be controlled in a more “extrinsic manner”.

TARGETD SIGNALS CONTROLLING AGE PHENOTYPE

Signaling pathways involving Notch, transforming growth factor- β (TGF- β), JAK/STAT, p38 mitogen-activated protein kinase (MAPK), oxytocin/MAOI and mTOR regulate tissue stem cell functions, and their changes with age could affect tissue maintenance and repair systems. Proper modulation of these pathways enhanced the tissue regenerative capacity of experimental animals.

Based on the fact that broad rejuvenation of aging by young systemic milieu has been shown in derivatives from all three germ layers, *i.e.*, muscle^[15], liver^[6] and brain^[16], as well as pancreas^[17] and heart^[18], it can be speculated that young blood serum and its chemical components may contain molecules involved in signals controlling the aging phenotype. However, only a few potential systemic factors responsible for this phenomenon have been identified, as the positive effects of young systemic milieu on old age are very limited^[11]. Further, aged systemic milieu or old serum can inhibit young tissue stem cell function^[15], suggesting inhibitory components may also exist in the aged circulatory system. Thus, removal or neutralization of these inhibitory systemic components would be necessary to rejuvenate tissue or cellular function.

In this regard, circulating factors that are increased or decreased in old animals represent potential targetable signals and pathways against aging. For instance, several TGF- β and Wnt signaling pathway effectors increased in older animals have been identified as pro-aging circulatory factors capable of deteriorating muscle regeneration^[15,19]. TGF- β and bone morphological protein pathways increase with age, activate p38 MAPK, and also act through SMADs. Inhibition of p38 MAPK and SMADs has been found to relieve some of the negative effects of pathogenic activation of these pathways occurring with age^[20].

JAK/STAT is a cytokine receptor pathway that increases with age. Many inflammatory cytokines

act through this pathway and its inhibition has been shown to restore stem cell symmetric expansion in muscle satellite stem cells^[21]. C-C motif chemokine 11 (CCL11) is also increased in elderly individuals, whereby it impairs neurogenesis and decreases cognitive capacity^[14].

Activation of Sirtuin family members is also related to rejuvenation, especially Sirtuin 6 (SIRT6), which is an important anti-aging factor in various cells. Downregulation of SIRT6 in bone marrow mesenchymal stem cells (BM-MSCs) impaired the proliferatory, migratory and oxidative stress resistance potentials of these cells. SIRT6 downregulation also enabled cellular senescence through increased senescence-associated β -galactosidase activity and p16 expression; although, SIRT6 is compensatorily overexpressed in aged BM-MSCs^[22].

In contrast, Delta/Notch signaling decreases with age, and activation of this pathway restores regenerative potential in old muscle^[23]. Oxytocin signaling also decreases with age, and restoring this signaling pathway has been shown to improve aged stem cell function in mesenchymal and muscle satellite stem cells through activation of the MAPK/extracellular signal-regulated kinase (ERK) signaling pathway^[24,25].

However, therapeutic modulation of these key pathways is not easy. For instance, long-term activation of the Notch signaling pathway or downregulation of TGF- β /SMAD and Wnt signaling pathways has been shown to be successful in rejuvenation; however, several side effects also occurred including oncogenic transformation, inadequate hematopoiesis and immune deregulation^[26]. Administration of oxytocin has also shown potential to stimulate malignant cell proliferation^[27].

One thing that must be emphasized is that these signaling pathways are highly interactive with each other. TGF- β acts through SMADs to influence downstream cytokine production that acts on the JAK/STAT pathway. SMAD3 and the Notch intracellular domain directly interact to form a nuclear complex capable of binding specific DNA sequences^[28]. The MAPK/ERK pathway is activated by oxytocin, as previously described, and the MAPK pathway is known to activate Notch signaling^[29]. Raf/MAPK/ERK and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling cascades also interact with several pathways through various mechanisms, such as crosstalk with TGF- β , Notch and Wnt pathways through Groucho/transducin-like enhancer of split or SMADs^[30,31]. PI3K/Akt signaling interacts with the Wnt pathway *via* the signaling molecule 14-3-3 η , which facilitates β -catenin activation by Akt and stabilizes the β -catenin complex to promote its nuclear translocation^[32]. This crosstalk of pathways with each other is largely mediated through mTOR signaling. Given that targeting these pathways is very complicated, further studies will be required to confirm the absence of safety issues prior to clinical applications.

PLASMA FRACTIONS/FACTORS CONTROLLING AGE PHENOTYPE

Regarding effects of the systemic environment on aging, as previously described, aged stem cells were rejuvenated by young plasma and young stem cells were aged by exposure to plasma from old animals^[23]. Thus, the presence of positive factors that promote young phenotypes in young plasma or negative factors that promote aging phenotypes in old plasma is speculated. Another possibility could be the presence of factors in young plasma that inhibit or neutralize "negative" or "aging" factors. For instance, naturally decreasing levels of interleukin 15 cause aging symptoms such as sarcopenia and obesity, suggesting this cytokine could be a "positive" factor for young phenotypes^[33]. In contrast, injection of CCL11/eotaxin, which is reduced by interleukin 15, into the systemic circulation of young animals caused a dysfunction in neurogenesis that resulted in brain aging and loss of cognitive function, suggesting this cytokine possesses pro-aging effects^[14].

Additional examples of aging factors include oxytocin and lamin A (specifically, progerin, a truncated form of lamin A). As previously described, oxytocin signaling decreases with age; however, restoring this signaling pathway improved the function of aged mesenchymal and muscle satellite stem cells through activation of the MAPK/ERK signaling pathway, suggesting oxytocin could be a systemically acting anti-aging molecule^[24,25]. Whereas, experimental induction of progerin reduced the regenerative capacity of cells by significantly disrupting the expression and localization of self-renewal markers, in part by deregulating Oct1, which perturbs both mTOR and autophagy pathways^[34,35].

Another example of concern is the case of growth differentiation factor 11 (GDF11); however, age-related levels of GDF11 and its function have generated an apparent controversy. Sinha *et al*^[36] argued that systemic GDF11 levels normally decline with age, and supplementation of GDF11 reversed functional impairments and restored genomic integrity in aged muscle satellite stem cells. Increased GDF11 levels in aged mice also improved muscle structural and functional features, and increased strength and endurance exercise capacity. However, Eggerman *et al*^[37] claimed that there was a trend toward increased GDF11 levels in the sera of aged rats and humans, and GDF11 mRNA also increased in rat muscle with age. They argued that GDF11 and myostatin both mechanistically induce SMAD2/3 phosphorylation, inhibit myoblast differentiation, and regulate identical downstream signaling. GDF11 significantly inhibited muscle regeneration and decreased muscle satellite stem cell expansion in mice. Thus, they concluded GDF11 could be a target for pharmacologic blockade to treat age-related sarcopenia.

One conclusion from these observations is that rejuvenation might require the presence, absence or a required concentration of a number of different factors,

such that a cell placed in a young or old environment could assume the age phenotype appropriate to that environment. However, conclusions regarding whether an increase in positive factors, decrease in negative factors (possibly by dilution in young plasma), or their combination results in rejuvenation is still under investigation. Hopefully, further elucidation of the molecular mechanisms underlying aging and rejuvenation will narrow the search so researchers can focus on not only investigating serum or plasma fractionation, but also molecules and agents that affect the aging/rejuvenation process.

POSSIBILITY OF ORGANISMIC REJUVENATION

As previously described, cross-age transplantation studies and parabiosis experiments revealed that the environment provided by young blood or plasma is capable of rejuvenating aged cells *in vivo*, and young plasma is sufficient to rejuvenate old stem cells *in vitro* or *vice versa*, *i.e.*, old plasma accelerates aging of young cells. Therefore, stem cells assumed the age phenotype of the "age environment" they are in, *i.e.*, either young or old. As such, aging could be caused by an accumulation of negative factors (aging factors) or by a decrease of positive factors (youth phenotype-promoting factors). Examples of negative (pro-aging) factors are p16INK4a, TGF- β and TNF- α , and positive (anti-aging) factors are Notch/Delta and Wnt pathways. We will discuss about the details later in this section.

In this context, organismic rejuvenation is potentially achieved by either removing deleterious substances from old plasma and stem cell niches, or by providing factors that promote young phenotypes in old plasma; indeed, both might be beneficial. In cross-age organ transplantation, the recipient organ experiences an environment that is entirely young or old, although, this method might not be clinically feasible.

If aging is a programmed process coordinated by plasma-borne factors, then exposing cells to the plasma of a particular age should make those cells exhibit a corresponding age phenotype in terms of gene expression profiles. Thus, it is considered that organismic rejuvenation could be achieved by exchange of as much blood or plasma as possible to reduce the effects of original blood or plasma, *i.e.*, heterochronic plasma exchange. Experimentally, the presence of positive, youth-promoting factors in young plasma has been demonstrated to rejuvenate neurogenesis and cognitive function by mere injection of young plasma into old mice^[38]. This suggests plasma replacement could be a treatment option for age-related diseases including dementia. The problem is that it is necessary to neutralize or remove inhibitory components occurring within the aged circulation in order for small volumes of young plasma to effectively enhance tissue regeneration in the elderly. Additionally, it is unclear which levels

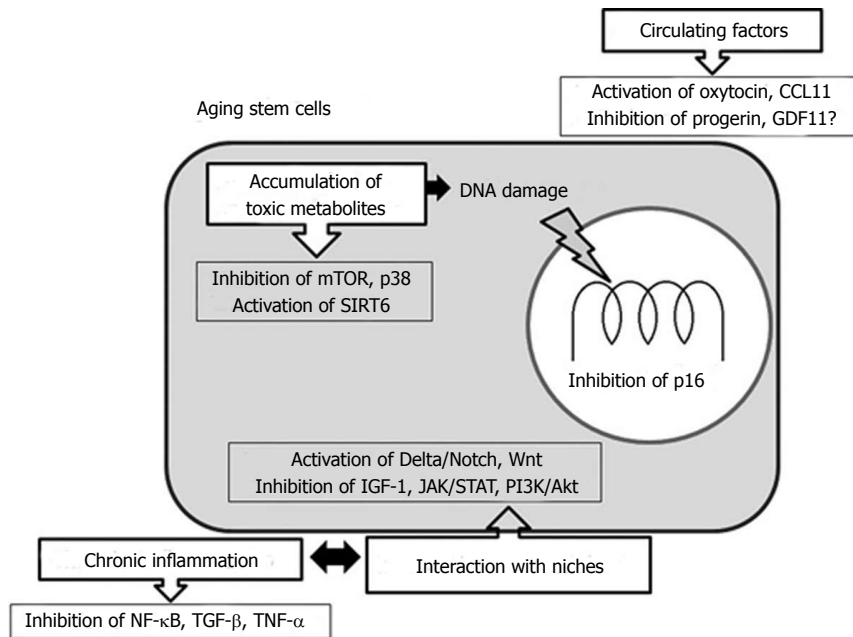


Figure 1 Possible targets for rejuvenating strategy in aging stem cells. Which cell types (*i.e.*, stem, progenitor, cycling and/or senescent) are capable of being rejuvenated and what modulation of signaling pathways can produce such cellular rejuvenation must be identified to make stem cell rejuvenation feasible. mTOR: Mammalian target of rapamycin; NF- κ B: Nuclear factor- κ B; TGF- β : Transforming growth factor- β ; TNF- α : Tumor necrosis factor- α .

of circulatory molecules are necessary and sufficient for pro-regenerative activity to occur in old stem cells. However, plasma from young animals should have sufficient factors/molecules for all signaling pathways described above to rejuvenate all stem cell types. Another potential issue could be that most or many of the important signaling molecules associated with age phenotype determination may have short half-lives, and would therefore be easily replaced by molecules from the recipient's body.

An issue of greater concern is the stem cell niches, as the age of stem cells appears to be determined by the age of their niche or environment, rather than the age of the stem cell for many tissues, such that young local and/or systemic environments promoted effective regeneration of old stem cells^[11]. Additionally, homeostasis of stem cell tissue maintenance and repair mechanisms would be regulated by the differentiated niches emanating signals, a feature that changes with age, such that niche cells also experience intrinsic aging, resulting in changed extrinsic influences on tissue stem cells. Considering these points, organismic rejuvenation must be performed by rejuvenating both stem cells and their niches. Most likely, rejuvenated niches could rejuvenate the stem cells already residing within them.

In that sense, plasma exchange, a replacement of plasma in an old body with plasma from a younger body, would be a potential means to rejuvenate old stem cells *in vivo*, although proper volumes and scheduling must be sufficient to allow cleansing of stem cell niches. The risks and costs of this process should also be weighed against potential benefits, as the effectiveness as well as safety issue of plasma exchange in rejuvenating stem cells has not yet been examined

in humans. Thus, there remains a major hurdle to applying this technique in clinical settings.

Molecularly, activation of Notch/Delta and Wnt pathways, and inhibition of TGF- β and TNF- α are all restored to the aged muscle niche to rejuvenate muscle satellite stem cells^[11], and down-modulation of mTOR rejuvenates hematopoietic stem cells^[3]. Exposure to youthful circulation, especially CD45⁺ hematopoietic cells, modulates Wnt/ β -catenin signaling to rejuvenate bone repair capacity^[39]. Insulin/insulin-like growth factor 1 (IGF-1) signaling molecules that have been linked to longevity in mammals include daf-2, InR and their homologues. Inactivation of these corresponding genes has been shown to increase the life span of nematodes, fruit flies and mice^[40]. If it is possible to target these molecular pathways by synthetic agents or natural sources, stem cell rejuvenation will be more feasible than plasma exchange; indeed, some agonists or antagonists of specific signaling pathways have already been developed and approved by the United States Food and Drug Administration. For instance, with the synthetic agents, activation of Notch and MAPK by attenuation of JAK/STAT signaling rejuvenates myogenesis^[21]; whereas, a TGF- β inhibitor simultaneously rejuvenates myogenesis and hippocampal neurogenesis^[41,42]. Attenuation of mTOR with rapamycin could also be a multi-faceted anti-aging strategy against senescence-associated cell cycle arrest to enhance tissue regeneration^[43,44]. Attenuation of the IGF-1 pathway with metformin has shown life-extending potential^[39]. Varieties of natural products such as phytochemicals, flavonoids or other plant extracts have also shown anti-aging effects by targeting various pathways including NF- κ B, mTOR, IGF-1 and PI3K/Akt pathways. For instance, the polyphenols resveratrol and

curcumin and the flavonoid genistein could be potential therapeutic agents to target signaling pathways involved in aging. Curcumin targets NF- κ B, STAT3, PI3K/Akt^[45] and mTOR^[46] signaling; whereas, resveratrol targets PI3K/Akt signaling by downregulating cyclin-dependent kinase 2, cyclin D1, proliferative cell nuclear antigen, and Akt-ERK signaling^[47]. Effective inhibition of multiple pathways involved in the aging process, including NF- κ B, mTOR, IGF-1 and PI3K/Akt, can possibly be achieved by appropriately combining these chemicals to rejuvenate stem cell populations.

Although all stem and progenitor cell populations (not to mention their niches) might not be rejuvenated, the rejuvenation of some cell populations including muscle satellite cells, bone marrow stromal cells and hematopoietic stem cells, will benefit the elderly population by increasing their quality of life. Eventually, it may also result in the prevention of aging by increasing the duration of youthful health.

CONCLUSION

The population of elderly individuals is dramatically increasing worldwide; thus, the importance of extending healthy life expectancy has been emphasized, especially in the rapidly aging societies of many developed countries. Aging affects multiple signaling pathways and their crosstalk, and changes the interaction between stem cells and their niches. As described, recent advances in aging research have indicated the possibility of rejuvenation at cellular, tissue and organismic levels, and suggested that rejuvenation of tissue stem cells through modulation of specific pathways plays an important role in this phenomenon.

To make organismic rejuvenation effective, we must see which cell types (*i.e.*, stem, progenitor, cycling and/or senescent) are capable of being rejuvenated and what modulation of signaling pathways can produce such cellular rejuvenation. Among multicellular organisms with reparable or regenerative tissues, aging entails another feature that causes a gain of function that allows cells to inappropriately proliferate and subsequently acquire phenotypes with increased ability to proliferate, migrate, colonize and survive in ectopic sites, as well as evade attacks by host immune surveillance systems. Thus, aging is one of the major drivers of malignant transformation. In contrast, in aging and cancer development processes, a stress response termed "cellular senescence" may be linked to multiple pathogeneses of both degenerative and hyperplastic diseases. In this regard, cellular senescence is generally considered to be a potent anti-carcinogenic program, and hyperplastic or neoplastic transformation possibly involves a series of events that bypass the senescence process^[48].

Figure 1 illustrates the possible targets for rejuvenating strategy in aging stem cells. To make stem cell rejuvenation more feasible and achieve the prevention

or delay of aging, a better understanding of aging in terms of molecular signaling networks for cellular communication involved in tissue homeostasis, maintenance and repair mechanisms is still required.

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Optimizing stem cells for cardiac repair: Current status and new frontiers in regenerative cardiology

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Abstract

Cell therapy has the potential to improve healing of ischemic heart, repopulate injured myocardium and restore cardiac function. The tremendous hope and potential of stem cell therapy is well understood, yet recent trials involving cell therapy for cardiovascular diseases have yielded mixed results with inconsistent data thereby readdressing controversies and unresolved questions regarding stem cell efficacy for ischemic cardiac disease treatment. These controversies are believed to arise by the lack of uniformity of the clinical trial methodologies, uncertainty regarding the underlying reparative mechanisms of stem cells, questions concerning the most appropriate cell population to use, the proper delivery method and timing in relation to the moment of infarction, as well as the poor stem cell survival and engraftment especially in a diseased microenvironment which is collectively acknowledged as a major hindrance to any form of cell therapy. Indeed, the microenvironment of the failing heart exhibits pathological hypoxic, oxidative and inflammatory stressors impairing the survival of transplanted cells. Therefore, in order to observe any significant therapeutic benefit there is a need to increase resilience of stem cells to death in the transplant microenvironment while preserving or better yet improving their reparative functionality. Although stem cell differentiation into cardiomyocytes has been observed in some instance, the prevailing reparative benefits are afforded through paracrine mechanisms that promote angiogenesis, cell survival, transdifferentiate host cells and modulate immune responses. Therefore, to maximize their reparative functionality, *ex vivo* manipulation of stem cells through physical, genetic and pharmacological means have shown promise to enable cells to thrive in the post-ischemic transplant microenvironment. In the present work, we will overview the current status of stem cell therapy for ischemic heart disease, discuss the most recurring cell populations employed, the mechanisms by which stem cells deliver a therapeutic benefit and

strategies that have been used to optimize and increase survival and functionality of stem cells including *ex vivo* preconditioning with drugs and a novel "pharmacooptimizer" as well as genetic modifications.

Key words: Stem cell; Regenerative medicine; Cellular cardiomyoplasty; Preconditioning; Myocardial infarction; Heart failure; Viability; Paracrine activity; Transplantation; Pharmacooptimizer

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Core tip: Cell therapy has the potential to improve healing of the ischemic heart, to repopulate injured myocardium and restore cardiac function in ischemic and non-ischemic cardiomyopathy. However, one of the biggest impediments lessening clinical effectiveness of cell therapy is the poor viability, retention and functionality of transplanted cells. This review looks at various stem cell *ex vivo* preconditioning and reprogramming methods aimed at enhancing the therapeutic potential of stem cells for heart failure treatment.

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STEM CELL THERAPY FOR ISCHEMIC HEART DISEASE

Considering the elevated morbidity and mortality of ischemic heart diseases, there is a pressing need to develop new therapeutic solutions to reduce ventricular remodeling, improve cardiac function and prevent development of heart failure (HF) following myocardial infarction (MI). For many of the patients, heart transplantation is a last resort option and its use is limited due to the scarcity of available donors. Therefore, myocardial stem cell therapy or cellular cardiomyoplasty is an approach that aims at inducing neoangiogenesis and even generating new functional myocardium. Many preclinical studies have involved transplanting cells in the border region of the infarcted myocardium to improve vascular supply, increase or preserve cardiomyocytes and repair damaged ones, and based on many positive findings, cell therapy has long been proposed as a potential treatment for HF^[1-3]. However, recent clinical trials have reported much less remarkable results with meta-analyses indicating a mean increase in ejection fraction (EF) of approximately 3% to < 6%, with better results in patients with low EF, or if cell infusion is delayed at least 5 d after MI^[4-7]. Randomized trials have also shown that the composite end point of death, infarction, revascularization, is significantly decreased at

12 mo, others have reported sustained benefits up to 5 years with reduced death and infarct size, improved myocardial perfusion and global cardiac function, whereas some have not found any profound long-term clinical benefit thereby advocating for cautious optimism in regards to cell therapy^[5,8-10].

Clearly evidence shows there is much room for improvement that can only be achieved through the fundamental understanding of the stem cell biology and mechanisms for the therapeutic benefit afforded by these cells. We now understand that only a small portion of cells are retained in the myocardium and that their paracrine activity will promote cardiac repair through production of anti-inflammatory, pro-survival and angiogenic factors^[11]. Indeed studies have shown that injection of stem cell conditioned media rich in these factors improve cardiac repair in HF models^[12]. These factors are able to attenuate tissue injury, inhibit fibrotic remodeling, stimulate recruitment of endogenous stem cells and reduce oxidative stress^[13]. Therefore, cell therapy can be viewed as providing cellular units releasing paracrine mediators to promote a beneficial effect^[14]. This is true of course only if the cells are retained long enough and remain viable in the transplant environment for this to occur.

STEM CELLS USED IN REGENERATIVE MEDICINE

Stem cells possess the capacity for prolonged proliferation, multilineage differentiation as well as trophic functions which enables tissue and organ repair^[15-17]. Cell types used for cardiac repair include unfractionated bone marrow cells (BMCs) and mononuclear cells, mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs), skeletal myoblasts (SkMbs), cardiac progenitor cells (CPCs), fetal cardiomyocytes, and embryonic stem cells (ESCs)^[18-20]. Each cell type has its advantages and disadvantages for cell therapy applications. Therapeutic injection of stem cells into a host requires accurate cell selection based on differentiation potential, relative ease of isolation, availability in large quantities, *in vitro* expansion^[21,22]. These cells are isolated from various sources. For instance, SkMbs are isolated by skeletal muscle biopsies and expanded *in vitro*. EPCs have shown the greatest potential for angiogenesis^[23], can be isolated from the blood. Resident cardiac stem cells or cardiospheres could be isolated from biopsies, clonally expanded *in vitro* and differentiated into cardiomyocytes^[24]. Bone marrow contains a heterogeneous cell population that includes differentiated cells and stem cells, such as HSCs, MSCs and EPCs. Due to its relative ease of accessibility and processing, as well as its ability to transdifferentiate into myocardial or vascular cells, BMCs have been readily used in clinical trials. However, contradictory benefits have been reported mainly since

either unfractionated or sub-populations of BMCs with or without *in vitro* culture steps have been employed in various studies^[25,26].

MSC

MSCs are one of the best candidates for heart disease cell therapy due to their easy isolation, rapid expansion and safety^[27]. MSCs retain their growth potential over several passages^[28,29] and have the ability to differentiate into osteoblasts, chondrocytes, myocytes, fibroblasts, adipocytes and other mesenchymal phenotypes *in vitro* and *in vivo*^[28,30-32]. In addition, MSCs are also immune-privileged because they express low levels of MHC II compared with MHC I^[33]. They display immunosuppressive effects allowing successful allogeneic transplantation. Many reports have shown improved recovery of ventricular function following MI with transplantation of MSCs in animal models^[34] as well as an improvement in cardiac function and infarct size in human trials^[29,35-38].

The safety and feasibility of intra-coronary MSC infusion and intra-myocardial delivery during coronary bypass grafting in post-MI patients has been demonstrated^[33,39]. However, MSC-based therapy has the fatal limitation of poor viability of MSCs after cell transplantation^[31]. Only approximately 5% of transplanted MSCs survive for 14 d in the infarcted porcine heart^[40], whereas survival rate of human MSCs transplanted in an uninjured mouse heart is less than 0.5% at 4 d^[31]. Similar results were obtained from studies using different cell types. For instance, about 7% of SkMb, 15% of smooth muscle cells, and 6% of unfractionated BMCs survived at 3 d to 1 wk in infarcted animal hearts^[41-43]. Consequently, cell viability is likely a common barrier for any cell therapy approach for MI.

HEMATOPOIETIC AND ENDOTHELIAL PRECURSOR STEM CELLS

HSCs count for perhaps as few as 1:10000 bone-marrow cells and are known for their positivity for the CD34 cell surface marker. EPCs also residing in the bone marrow, have originally been defined by their expression of the CD133, CD34, and the vascular endothelial growth factor receptor-2 (VEGFR-2) markers. CD133 or prominin-1 is a highly conserved stem cell glycoprotein antigen described as marker for identification of early immature EPCs^[44]. CD133⁺ cells migrate upon gradients of vascular endothelial growth factor (VEGF) and stromal-derived factor (SDF) *in vitro* and *in vivo*^[45-47]. CD133⁺ cells *in vitro* differentiate into endothelial cells and release paracrine angiogenic cytokines. Differentiated CD133⁺ are capable of inducing capillary tubes *in vitro*^[46,48-51] and several clinical trials have reported promising effects following infusion or direct intramyocardial injection of autologous CD133⁺ cells into

ischemic hearts^[52-56].

TRANSPLANT CELL DEATH IN THE INFARCTED HEART

One of the prime challenges of stem cell therapy consists in the survival, retention and differentiation of cells delivered in the harsh microenvironment of diseased tissues or organs^[31,57-59]. Poor retention and survival of transplanted cells in the heart which can decrease to 39% at 1 h following injection as seen in human studies^[60-64] or reach at most 21% in animal models following intramyocardial injection^[65,66], further decrease exponentially thereafter due to apoptosis^[31,57,67,68]. The increased cell death is swayed by various inflammatory response mediators, mechanical injury, hypoxia and ischemia-reperfusion stressors, and influenced as well by the donor cell source and quality^[69]. Indeed, the cause of death of implanted cells may begin during the preparation step where MSCs for example, which are normally grown attached, are prepared in suspension in order to be injected. The loss of matrix attachments causes programmed cell death called "anoikis"^[69-73]. Adhesion of cells to the matrix predominantly *via* integrin molecules represses apoptotic signaling, whereas detachment has the opposite effect. This effect is compounded by the hostile microenvironment of diseased myocardium which includes deprivation of nutrients and oxygen, upregulation of inflammatory mediators and low pH leading to poor transplant survival^[70,74,75]. Moreover, myocardial injury generates an inflammatory response involving neutrophils and macrophages^[76] which themselves produce inflammatory cytokines and reactive oxygen species (ROS) that may intensify the inflammatory response and anoikis signals and lead to cell death as well^[77-79]. Indeed, co-injection of SkMbs with the ROS scavenger superoxide dismutase (CuZn-SOD) increases graft survival^[43].

MECHANISMS OF INFARCT REPAIR BY STEM CELLS: PARACRINE MODULATION OF ISCHEMIC ENVIRONMENT

Several studies have shown that recruitment of endogenous stem cells or their delivery to injury sites results in structural regeneration and functional improvement^[80]. While the original thesis regarding the beneficial mechanism pointed to stem cells and their differentiation within the host myocardium, we now understand that few if no exogenously administered cells engraft and differentiate^[81-84]. It is rather the paracrine biomolecules produced by stem cells which account for the bulk of observed functional repair and these molecules also reduce cell death in cardiomyocytes and other populations thereby benefiting the diseased host tissue^[85-89]. Stem cells secrete an array of cytokines, growth factors and extracellular

matrix (ECM) components that act in an autocrine or paracrine manner. Cytokines are signaling and immune-modulating agents involved in cellular communication, whereas chemokines also produced by stem cells are involved in chemotaxis, while growth factors stimulate cell growth, proliferation and differentiation. Moreover, antioxidants, anti-apoptotic, anti-inflammatory or immunosuppressive molecules also secreted by stem cells can protect the cellular niche and transplant micro-environment from damaging mediators such as ROS. Finally, angiogenic and antifibrotic factors secreted by stem cells are responsible for tissue repair. In view of the numerous bioactive molecules produced and secreted by stem cells, current research using transcriptomic and proteomic technologies is poised at identifying the precise beneficial mediators and developing ways to harness these powerful pathways and mechanisms of repair^[80,90-94].

The cardioprotective panel of stem cell secreted factors include bFGF/FGF-2, IL-1 β , IL-10, PDGF, VEGF, HGF, IGF-1, SDF-1, thymosin- β 4, Wnt5a, Ang-1 and Ang-2, MIP-1, EPO and PDGF^[21,85-89,95]. FGF-2 reduces ischemia-induced myocardial apoptosis, cell death and arrhythmias, and stimulates increased expression of anti-apoptotic Bcl-2^[96,97]. HGF, bFGF, Ang-1 and -2, and VEGF secreted by BMMSCs lead to augmented vascular density and blood flow in the ischemic heart^[91,98,99], whereas SDF-1, IGF-1, HGF facilitate circulating progenitor cell recruitment to injury sites thereby promoting repair and regeneration^[100-103]. Stem cells also secrete ECM components including collagens, TGF- β , matrix metallo-proteinases (MMPs) and tissue-derived inhibitors (TIMPs) that inhibit fibrosis^[104-106] and may thereby benefit cardiac tissue remodeling post-MI.

STRATEGIES TO ENHANCE STEM CELL SURVIVAL

It is clear that the injected stem cells must survive and thrive in the injured or diseased transplant environment for any significant repair to occur. Acute cardiac ischemia results in a hypoxic and inflammatory microenvironment which makes it extremely difficult for the injured area to be functionally repaired^[107-109]. Consequently the injected cells will need to be tolerant of these deleterious conditions^[110-113]. For this, *ex vivo* manipulation of cells has been used to overcome cell survival issues as well as to enhance metabolic characteristics in order to confer cells with a powerful advantage in the critical early days after transplantation. Preconditioning, or pre-treating and reprogramming cells by physical/environmental, pharmacological, genetic manipulations or with cytokine and growth factor treatments has shown great potential to prime cells to withstand the rigors of the transplant microenvironment post-ischemia and maximize the cells' biological and functional properties. In addition, there are strategies to modify the transplant environment through immune

modulation and even by increasing cell retention with bio-scaffolds.

PRECONDITIONING STEM CELLS USING PHYSICAL/ENVIRONMENTAL CHALLENGES

Beneficial effect of preconditioning was first demonstrated by treating healthy heart with intermittent cycles of non-lethal ischemia followed by reperfusion. This manipulation protected the myocardium from a subsequent important ischemic episode^[114]. Subsequently, various strategies including hypoxic, oxidative and thermal conditioning challenges have been studied in an attempt to improve stem cell survival^[115-118]. Low oxygen culture conditions (0.5% O₂ for 24 h) have been shown to trigger survival pathways in MSCs before their engraftment *in vivo*^[119]. MSCs exposed to hypoxia *in vitro* showed upregulation of Bcl-2 and Bcl-XL survival genes, promoting reduced infarct size and enhanced cardiac function^[119]. Hypoxia preconditioning also increases *in vitro* expression of antiapoptotic genes such as Akt and eNOS^[81,88,116]. Hypoxia treated cells show significantly improved survival post-engraftment in the infarcted heart^[119]. Also, during ischemic preconditioning, hypoxia inducible factor-1 α (HIF-1 α), a master regulator of genes responsible for low oxygen survival signaling^[119-121], stimulates the transcription of VEGF and erythropoietin that increase cellular oxygen availability by promoting angiogenesis and erythropoiesis^[122,123]. In addition to VEGF, temporary exposure to hypoxia increases expression of many growth factors including bFGF, HGF, IGF-1, and thymosin- β 4^[124,125] which are implicated in cell mobilisation and apoptosis.

In addition to promoting pro-survival and cytoprotective effects, hypoxic preconditioning supports cells to preserve their stemness and promote their differentiation and proliferation potential post-engraftment^[116,126-129]. Furthermore, BMMSCs exposed to anoxic conditions and transplanted into infarcted myocardium have been shown to exert increased protective effects on cardiomyocytes^[130]. Thus, hypoxic treatment may lead to enhanced donor and host cell survival in ischemic environments and provide functional benefits.

Burst exposure to low levels of oxidative stress *in vitro* also increases stem cell viability as seen for example by the exposure of CPCs *in vitro* to low concentration of H₂O₂ prior to implantation in ischemic rat hearts^[131]. Similarly, NPCs exposed to non-cytotoxic low dose treatment of H₂O₂ demonstrated improved resistance to lethal oxidative stress^[132], and MSCs preconditioned with H₂O₂ and transplanted in the ischemic heart display increased viability and functional improvement^[133].

Heat shock treatment is also an interesting approach to enhance cell survival. Heat shock protein (HSP) generation can be achieved by exposing cells to elevated temperatures (39 °C to 45 °C). Thermal shock

of primary cardiomyocytes has been shown to result in increased expression of HSP70 thereby protecting the cells from *in vitro* and *in vivo* oxidant stress^[134,135]. Transplantation of human ESC-derived cardiomyocytes treated by 30 to 60 min of 43 °C heat upregulates HSPs such as HSP60, 70, and 90 has been shown to improve graft functionality in a rat model of MI injury^[136,137]. Exposing MSCs to elevated temperature (43 °C) also induces secretion of HSPs, including HSP27 and HSP70^[138] which may contribute to increased cell survival. Similarly, culture of CPCs at 42 °C has been shown to reduce apoptosis, increase functionality, and reduce fibrosis of mouse ischemic myocardium^[139]. Considering the role of HSPs in cell protection and immune modulation, thermal conditioning represents an easy and effective means of increasing cell viability, retention and consequently improving stem cell graft function.

PRECONDITIONING STEM CELLS WITH DRUGS

The effectiveness of preconditioning on cell viability and function can also be achieved by pharmacological treatments^[118]. Other than the initiation of survival signaling, treating cells with conditioning mimetics causes release of growth factors and cytokines that exert protective and angiomyogenic effects. Preconditioned cells show greater release of growth factors including VEGF, Ang-1, SDF-1 α , HGF, and IGF^[118]. Several drugs including mitochondrial potassium channel openers that promote influx of K⁺ through ATP-sensitive K⁺ channels (mitoKATP) are useful agents altering the apoptotic cascade by preventing cytochrome c release^[140-143]. Pinacidil or Diazoxide, well-known non-selective mito-KATP channel openers have been demonstrated to suppress apoptosis^[144-146]. SkMbs and BMMSCs treated with Diazoxide demonstrated increased cell survival in ischemic environment, and increased secretion of Ang-1, bFGF, HGF and VEGF by preconditioning was proposed to augment angiomyogenesis^[146,147].

HMG CoA reductase inhibitors (Statins) appear promising in blocking apoptosis, prolonging stem cell survival and improving organ repair. Treatment with atorvastatin for example enhances cell survival and differentiation into cardiomyocytes, decreases the infarcted area, promotes angiogenesis, and reverses the ventricular remodeling processes^[148]. Also, *ex vivo* statin treatment has been shown to prevent impairment of the functionality of EPCs *in vitro* as well as the loss of telomere repeat-binding factor 2, whose expression is reduced in end-stage human HF, and functions to prevent cells from entering in apoptosis or senescence^[149,150]. A recent review provides encouraging basis for the use of statins to increase the number and/or function of MSCs and EPCs for cell therapy^[151].

Preconditioning cells with naturally occurring hormones such as Oxytocin (OT) or its synthetic analog

drug (Pitocin) is another means for stem cell optimization. Indeed, OT preconditioning of various cell types makes them resistant to oxidative stress^[152], and primes stem cell differentiation into cardiomyocytes^[153] and vascular cells^[154]. MSC express a functional OT receptor which mediates glucose uptake^[155] and cell differentiation^[156] it has been shown that OT modulates gene expression for adhesion molecules and MMPs involved in cellular migration^[154,157,158]. Our group showed that OT treated MSC respond with rapid calcium mobilization and upregulation of the protective pAkt and pErk1/2 proteins. Functional analyses revealed the involvement of these kinase pathways in cell proliferation, migration, and protection against apoptotic effects of hypoxia and serum starvation. OT preconditioning increased upregulation of genes with angiogenic, anti-apoptotic and cardiac anti-remodeling properties such as HSP27, HSP32, HSP70, VEGF, thrombospondin, TIMPs and MMPs, and co-culture of cardiomyocytes with OT-preconditioned MSC reduced apoptosis^[159].

Various other classes of drugs and chemicals have also shown potential for use as stem cell *ex vivo* conditioning agents. Treatment of BMMSCs with trimetazidine (1-[2,3,4-trimethoxybenzyl] piperazine), an anti-ischemic drug for angina treatment has been shown to increase cell viability in response to oxidative stress^[160]. Also, treatment of rat BMMSCs with β -mercaptoethanol was shown to upregulate HSP72 resulting in improved resistance to oxidative injury^[161]. Also, the pan caspase inhibitor ZVAD-fmk has been shown to increase engraftment of HSC during intra-bone marrow transplantation procedure in allogeneic mice^[162]. This said, one has to be mindful of the balance between enhancing stem cell survival and enabling unintended carcinogenic effects when selecting compounds in the development of stem cell conditioning agents.

Finally, a means to favor stem cell differentiation would constitute an interesting pharmacological conditioning method for improving graft function. Small molecules such as 5-Azacytidine, a DNA demethylating agent^[32], have been shown to prime cardiac differentiation in MSCs. Other molecules including the HSP90 inhibitor Geldanamycin^[163], the kinase inhibitor Imatinib Mesylate^[164] and the proteasome inhibitor Bortezomid^[165] have been shown to instruct stem cell commitment to various lineages.

A NOVEL STEM CELL PHARMACO-OPTIMIZER

Stem cell "pharmaco-optimization" as we term it, is the process of contacting stem cells *ex vivo* with drugs in order to enhance their innate therapeutic qualities and develop a desirable phenotypic profile with enhanced cellular functions and viability favored in the context of stem cell therapy.

Celastrin is an antioxidant molecule extracted from the root of a vine (*Tripterygium wilfordii*) which has showed beneficial effects in the treatment of various diseases including cancer, neurodegenerative diseases, autoimmune diseases, and inflammatory conditions^[166-171]. We are the first to report Celastrin's efficacy as a potent infarct sparing agent^[172] and we propose its use as a stem cell pharmaco-optimizer considering in part Celastrin's targeting and activation of two very potent cellular defence mechanisms: The heat shock response (HSR) and the antioxidant response (AR). HSR leads to cell protection against various physiological stresses^[173,174] via activation of HSP. HSR is regulated at the transcriptional level by the activation of heat shock factors with heat shock factor 1 (HSF1) being the master switch for HSP expression^[174]. The AR is mediated by the transcription factor nuclear factor (erythroid-derived 2)-like 2 (NRF2). NRF2 is a key controller of the redox homeostatic gene regulatory network including antioxidant proteins and phase II enzymes such as glutathione S-transferase, heme oxygenase 1 (HO1), NADPH-quinone oxidoreductase 1, superoxide dismutase 1-3 (SOD1-3), catalase (CAT), thioredoxin, glutathione peroxidase (GPx), and non-enzymatic antioxidants such as glutathione which exert protective, antioxidant, and anti-inflammatory effects^[173-176]. Under homeostatic conditions, HSF1 is bound and silenced by its natural repressor HSP90 chaperone, and NRF2 is similarly repressed by KEAP1 (Kelch-like ECH-associated protein1). During oxidative and electrophilic stress (ROS increase), NRF2 is liberated from KEAP1 and binds to antioxidant response elements in the promoter region of genes including HO-1. Similarly, during cellular stress, HSF1 translocates to the nucleus where it binds to heat shock elements as a phosphorylated-trimer and drives the transcriptional activity of HSPs^[177].

Briefly, Celastrin targets the interaction between HSP90 and its essential cofactors (*i.e.*, Cdc37)^[178], and through HSP90 functional inhibition, Celastrin promotes HSF1 release and HSR activation. Similarly, through a ROS/KEAP1/NRF2 pathway Celastrin activates the AR^[179]. Together, Celastrin activates the two evolutionary conserved cellular protective mechanisms as detailed above and is able to stimulate a powerful endogenous protective effect that could be harnessed to increase viability and therapeutic efficiency of stem cells.

PRECONDITIONING STEM CELLS WITH GROWTH FACTORS AND CYTOKINES

Pre-treating stem cells with growth factor (GF) is a simple and safe strategy to improve cellular survival, proliferation and differentiation. For example, preconditioning EPCs by culturing them in medium supplemented with VEGF, activates Akt and significantly reduces apoptosis in a dose-dependent manner^[180]. Also, by exploiting the SDF-1/CXCR4 ligand/receptor interaction which modulates cell growth, proliferation,

survival, migration and transcriptional activation^[21,181-184], SDF-1 can be used as a preconditioning chemokine^[185]. Indeed, treatment with recombinant SDF-1 enhanced vascular density and survival of cells under anoxic condition *in vitro* and following engraftment in the infarcted heart^[185]. Also, it has been shown that IGF-1 preconditioning of bone marrow-derived Sca-1⁺ cells upregulates connexin 43 which improves survival and integration of cells with host myocytes^[186]. The anti-apoptotic effects of IGF-1 are mediated by IGF-1/IGF-1R ligand/receptor interaction which involves PI3K/Akt and MAPK/Erk1/2 activation, whereas knockdown of connexin 43 rescinds cell viability to hypoxia *in vitro* and *in vivo* in the infarcted heart.

An additional strategy may consist of preconditioning cells with anti-inflammatory cytokines such as interleukin-10 (IL-10) which promotes multiple effects including down-regulation of Th1 cytokines such as IL-2, IFN- γ , TNF- α , and increase expression of the cell survival gene Bcl-2 thereby increasing stem cell survival^[187]. It also has been demonstrated *in vitro* and *in vivo* that in the presence of IFN- γ , MSCs suppress T-cells and graft vs host disease^[188-190].

EX VIVO GENETIC OPTIMIZATION OF STEM CELLS

Survival, differentiation and angiogenesis as targets

Stem cells are excellent vehicles for therapeutic gene delivery and can be genetically engineered for gene overexpression. Transgenes can encode for a myriad of beneficial factors including angiogenic and chemoattractant factors, anti-apoptotic proteins and growth factor(s) of interest^[181,191-193] and serve as a continuous source for these to mediate sustained intracrine, autocrine, and paracrine effects. Indeed, molecules secreted by transgene-modified MSCs may have different therapeutic profiles compared with normal MSCs. For example, transformation of stem cells to overexpress IGF-1 promotes donor cell survival, engraftment, and differentiation in cardiac cell therapy^[194-196]. IGF-1 induces expression of the pro-survival genes PI3-kinase, Akt, Bcl-xL and SDF-1 which is a potent chemoattractant of stem cells. Indeed, IGF-1 transformed MSC improve EF and fractional shortening in an infarct model^[197]. Cells have also been manipulated to overexpress Ang-1, HGF, VEGF and MyoD for post-MI myocardial repair. Results show increased cell engraftment, angiogenesis and commitment to the myogenic lineage in the ischemic region^[100,198-205]. Indeed, any therapeutic approach aimed at increasing vascularization within the ischaemic heart tissue will improve functional repair and recovery of the infarcted myocardium. One of the key proteins is VEGF whose overexpression will promote a strong pro-angiogenic signal. VEGF has been shown to promote endothelial cell survival^[206,207], and myocardial transfer of VEGF-transfected MSCs lead to better improvement of myocardial perfusion and heart function following

ischemia^[192,208]. Studies evaluating other angiogenic and myogenic genes with various VEGF isoforms, PDGF and TGF- β 1, have also suggested enhancement of cell therapy efficacy^[209]. VEGF is itself regulated by the transcription factor HIF-1 α which plays a critical role in the stabilization of VEGF transcription during hypoxia^[210,211]. Therefore, HIF-1 α overexpression has also been evaluated as a means to optimize BMMSCs for increased VEGF expression^[212].

Stem and progenitor cells have also been engineered to survive and engraft more effectively in hostile environments^[213,214]. Transfection of MSCs with growth factors such as bFGF shows increased survival in hypoxic conditions. These transformed cells also improve neovascularization compared to non-transformed MSCs^[215]. Interestingly, Akt-modified BMMSCs exhibit resilience to apoptosis through secretion of growth factors such as bFGF, HGF, IGF-1 and VEGF, as well as secreted frizzled-related protein 2 (Sfrp2) which exerts a beneficial effect on the infarcted heart post-engraftment by antagonizing pro-apoptotic properties of Wnt3a. Together, secretion of these factors known to exert pro-angiogenic, cardioprotective and inotropic actions^[125] is increased under hypoxic conditions^[81,125,216]. Transplantation of Akt-modified BMMSCs in the infarcted myocardium safeguards surviving myocardium for up to 2 wk post-MI at least in part through paracrine actions^[217]. In another study, MSCs overexpressing Akt with Ang-1 provide long-term therapeutic benefits for preventing apoptosis in an ischemic heart up to three months after initial transplantation^[218]. This said, it is interesting to note that medium from BMMSCs overexpressing Akt cultured under hypoxic conditions show an increase of many beneficial molecules including VEGF, FGF-2, HGF, IGF-1, and TB4, and trigger an increase in contractile response of cultured rat cardiomyocytes as well as improves ventricular function in a rat infarction model^[125]. In addition to Akt overexpression, BMMSCs have been engineered with anti-apoptotic genes such as Bcl-2 and HO-1. Bcl-2 overexpression in BMMSC decrease apoptosis of BMMSCs and increases VEGF secretion and capillary density in the infarct border zone thereby increasing functional recovery in ischemic myocardium^[124]. HO-1 exerts potent antioxidant and cytoprotective activity in the ischemic environment^[219,220]. HO-1 transfected MSCs are resistant to apoptosis and inflammatory injury and display improved tolerance to ischemia-reoxygenation injury harsh transplant microenvironments^[221]. Another opportunity to enhance transplanted cell survival in the damaged heart is to transfect them with recombinant HSPs, that represents a family of inducible and constitutively expressed proteins responsible for potent increase in cell tolerance to environmental stress including ischemia, hypoxia, oxidative injury, heat stress, and ischemia-reperfusion injury^[222]. Indeed, cells transfected with HSP encoding genes, namely HSP70, are protected from ischemic injury *in vitro* and *in vivo*^[223-226].

In order to procure a holistic coverage of survival and growth effects, combination treatment of stem and progenitor cells can be achieved prior to their transplantation. As mentioned, combined overexpression of Akt and Ang-1 has been attempted in MSC. Ang-1, a potent modulator of vascular development activates survival signaling^[227-229], and co-expression with Akt was shown to be more effective for cytoprotection in the context of lethal anoxia^[230]. Simultaneous overexpression of Akt and Ang-1 in MSC transplanted in infarcted rat heart conferred better engraftment, and cells were able to adopt myogenic and endothelial phenotypes. Combination treatments may also be more ambitious by including various components such as a collagen matrix (matrigel) to increase retention and prevent anoikis, Bcl-xL and Cyclosporine A to block mitochondrial death pathways, an inducer of mitoKATP channel opening such as Pinacidil or Diazoxide to mimic ischemic conditioning, a caspase inhibitor such as zVAD-fmk and IGF-1 to activate Akt pathways as previously described^[136].

Adhesion as a target

Adhesion is necessary for cell survival and is a key factor for MSC differentiation. Disruption of cell-ECM contact with trypsinization may facilitate apoptosis once cells are transplanted. Therefore, over-expression of adhesion molecules may enhance cell retention and improve viability. For example, tissue transglutaminase (tTG) over-expression in MSC leads to increased survival via an integrin-dependent mechanism^[231]. tTG also acts as a coreceptor for fibronectin (Fn)^[232,233] and enhances adhesion by bridging integrins and Fn or by mediating formation of ternary complexes^[234]. Compared to simple MSC transplantation, tTG transformed MSCs have been shown to better restore cardiac function of infarcted myocardium^[231]. Also, transfection of the integrin-linked kinase (ILK), a 59-kDa Ser/Thr kinase that binds to the cytoplasmic domain of β -integrin and participates in cell adhesion, growth, and ECM assembly, activates Erk and Akt phosphorylation which play important roles in cell survival during hypoxia^[77,235-238]. Transplantation of ILK-MSCs has been shown to further reduce infarct size, improve left ventricle function and increase microvessel density^[239].

Stem cell rejuvenation as a target

Increasing evidence supports the concept of senescence affecting tissue resident stem cells and diminishing regenerative capacity of organs^[240-242]. Cellular senescence is induced by multitude of stressors including hypoxia and oxidative conditions^[243-245] which reduces the cell's proliferative, differentiation and metabolic potential, and upregulates apoptotic markers^[246-255]. At the genomic level, aging appears associated with increase in p53-associated genes in addition to modulation of telomere, mitochondrial and apoptotic process^[255,256]. These age related changes limit the

ability of stem cells to secrete angiogenic factors thereby reducing their regenerative potential. It has been shown that MSCs from old patients are less effective in preventing ventricular remodelling and inducing new vessel formation post-MI^[248]. Old donors exhibit reduced tolerance to ischemia and decreased transplant survival within ischemic muscle^[251]. Similarly, older recipients have a diminished therapeutic response to receiving stem cells from donors of any age^[251,254]. To overcome these effects related to cellular senescence, many strategies are being developed as recently reviewed^[257]. In this regard, modifications to improve regenerative capacity have been sought^[30,81,258,259] and include genetic modification of human CPCs with Pim-1, a pro-survival downstream effector of cytokine signalling pathways^[260] including Akt^[261], in order to improve cellular metabolic activity^[262]. The WNT/ β -catenin pathway has also been studied as a potential target for MSC rejuvenation^[263]. While increasing age is associated with reduced MSC proliferation, differentiation capacity and WNT/ β -catenin signalling, lithium treatment which increases β -catenin bioavailability restores the impaired function of these cells^[257].

CONCLUSION

The use of stem cells to regenerate heart muscle has revolutionized the clinical practice for ischemic heart disease treatment. While safety and feasibility of cell therapy has been demonstrated in experimental and clinical studies, and the technology is making its way from bench to bedside, in order to reap the full regenerative potential afforded by stem cells, there is a necessity to develop the tools and the understanding required to ameliorate clinical efficacy. Most importantly, in order to harness the full therapeutic potential of these cells for cell therapy or any regenerative medicine application, optimization of cell viability, retention and functionality are of utmost importance. As summarized here, many groups are currently investigating various avenues of stem cell optimization. These methods include cell preconditioning using environmental stressors, genetic manipulations to enhance survival pathways, increase angiogenesis and cell adhesion, as well as preconditioning methodologies involving *ex vivo* stimulation of stem cells with growth hormones, cytokines and pharmacological agents such as statins and conditioning mimetics. The latter pharmacological method may be one of the safest, quickest, most reproducible, reliable and readily transferable method to the clinic used for producing optimized cell populations for patients. It is also foreseeable that in order to further enhance the therapeutic quality of these cells, multiple cellular pathways and effectors may be targeted, drug cocktails may be developed, or even conditioned cells may be combined with hydrogel technologies to encapsulate cells in a favorable environment to further promote retention, limit anoikis and facilitate cell-cell

and cell-matrix interactions. All of these upcoming advances in stem cell optimization will greatly benefit patients and the promising field of regenerative medicine in the coming years.

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Regulation of mitochondrial function and endoplasmic reticulum stress by nitric oxide in pluripotent stem cells

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Abstract

Mitochondrial dysfunction and endoplasmic reticulum stress (ERS) are global processes that are interrelated and regulated by several stress factors. Nitric oxide (NO) is a multifunctional biomolecule with many varieties of physiological and pathological functions, such as the regulation of cytochrome c inhibition and activation of the immune response, ERS and DNA damage; these actions are dose-dependent. It has been reported that in embryonic stem cells, NO has a dual role, controlling differentiation, survival and pluripotency, but the molecular mechanisms by which it modulates these functions are not yet known. Low levels of NO maintain pluripotency and induce mitochondrial biogenesis. It is well established that NO disrupts the mitochondrial respiratory chain and causes changes in mitochondrial Ca^{2+} flux that induce ERS. Thus, at high concentrations, NO becomes a potential differentiation agent due to the relationship between ERS and the unfolded protein response in many differentiated cell lines. Nevertheless, many studies have demonstrated

the need for physiological levels of NO for a proper ERS response. In this review, we stress the importance of the relationships between NO levels, ERS and mitochondrial dysfunction that control stem cell fate as a new approach to possible cell therapy strategies.

Key words: Endoplasmic reticulum stress; Mitochondrial function; Nitric oxide; Pluripotency; Cell differentiation; Mitochondrial biogenesis

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Core tip: Several studies have focused on the role of nitric oxide (NO) in regulating many physiological functions, such as metabolism and pluripotency. NO has been established to act as a potent agent for the control of stemness by promoting the expansion of pluripotent cells. NO regulates mitochondrial function and endoplasmic reticulum stress. In pluripotent stem cells, both of these factors are related to the control of cell fate and may contribute to the mechanism by which NO regulates the maintenance of pluripotency. This provides additional evidence supporting the use of NO as an alternative small molecule for the conservation and expansion of cultured pluripotent cell lines necessary for implementing a cell therapy programme.

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INTRODUCTION

Actions of nitric oxide in cells

Nitric oxide synthase (NOS) modulates the L-arginine-to-L-citrulline pathway, by which nitric oxide (NO) is synthesized^[1,2]. NO, a short-lived free radical, reacts with oxygen, superoxide, cytochrome c oxidase (CcO) and other molecules when it acts independently of cyclic guanosine monophosphate (cGMP), and this interaction is dose-dependent. Therefore, at high levels of NO, reactive oxygen species and NO interact to contribute to protein posttranslational modifications through S-nitrosylation and S-nitration^[3-5]. When it acts in concert with cGMP, NO activates soluble guanylate cyclase (sGC), catalysing the conversion of GTP into cGMP, which controls a variety of physiological effects in multiple tissues^[6,7]. The role of NO/cGMP in embryonic development and cell differentiation is a subject of intensive investigation; however, it is not clear whether the action of NO in stem cell biology is mediated *via* the cGMP pathway. It has been reported that bone marrow stem cell potency and differentiation are independent of the sGC/cGMP pathway^[8-12].

Moreover, many effects of NO on stem cell pluripotency and differentiation are independent of this pathway, and thus the mechanism by which NO modulates the differentiation of embryonic stem cells (ESCs) remains unclear^[13,14].

Physiological functions of NO

NO has been described to have important roles as a regulator of multiple physiological functions: It is a principal mediator of the immune system in the inflammatory response and a neurotransmitter in the central nervous system; acting as a second messenger, it has multiple biological effects that have been implicated in numerous physiological functions in mammals, such as regulation of blood pressure *via* smooth muscle relaxation and inhibition of platelet aggregation^[13,15-17]. NO has been reported to affect gene expression at the levels of transcription and translation and has been associated with the regulation of cell survival and proliferation in diverse cell types^[14,18]. Moreover, important processes such as growth, survival, proliferation, differentiation, and the pathologies of various diseases such as cancer, diabetes, and neurodegenerative diseases are mediated by functions of NO^[19,20]. NO has also been shown to be involved in the control of heart functions and cardiac differentiation/development^[21,22].

NO is considered one of many molecules that act on specific cell signalling pathways involved in embryonic development, specifically playing a dual role in the control of ESC differentiation and morphogenesis^[23].

This dual role is determined by the NO concentration: It has been demonstrated that a low NO concentration maintains pluripotency, whereas a high concentration induces differentiation^[14,18,23]. A high NO concentration has been reported to cause oxidative and nitrosative stress and apoptosis, processes partly responsible for cell death during chronic and degenerative disease. Moreover, embryonic stem cell differentiation is promoted by pharmacological treatment with high NO concentrations^[18,24,25]. Our group has reported^[18] that exposure to high concentrations of a NO donor (DETA-NO) promotes the differentiation of mouse ESCs induced by down-regulation of the pluripotency genes *Nanog* and *Oct4*. However, low NO concentration has been shown to promote cell proliferation and survival. Specifically, our group has demonstrated that the exposure of ESCs to a low concentration of DETA-NO promotes the expression of self-renewal genes and prevents the differentiation of mouse and human ESCs^[14]. Therefore, NO is also considered a regulator of cellular respiration (oxygen-sensitive pathways) and metabolism, with a major role in regulating the hypoxia response by modulating the activity of CcO, a component of complex IV, which is involved in the final processes of the mitochondrial electron transport chain^[4,16,26-28].

High NO

NO is a small molecule that has dual roles in the control

of ESC differentiation and tissue morphogenesis. High concentrations of NO promote differentiation. It has been reported that in ESCs, the production of NO is necessary for cardiomyogenesis because the maturation of terminally differentiated cardiomyocytes is prevented by NOS inhibitors^[22]. Mouse ESC (mESC) differentiation is promoted by high DETA-NO concentrations, which induce the down-regulation of *Nanog* and *Oct4* expression. NO represses *Nanog* via the activation of p53, which is associated with covalent modifications such as Ser315 phosphorylation and Lys379 acetylation. Moreover, the expression of the definitive endoderm markers *FoxA2*, *Gata4*, *Hfn1-β* and *Sox17* is increased by exposure to high concentrations of DETA-NO^[8,18]. It has been reported that the NO concentration regulates signalling pathways implicated in the survival and homeostasis of RINm5F cells. Thus, high NO can cause oxidative and nitrosative stress and apoptosis^[18,24,25]. Several studies report that NO induces apoptosis in various cell types, such as pancreatic beta cells^[29,30], thymocytes^[31] and hepatocytes^[32]. In ESCs, high levels of DETA-NO promote nitrosative stress, inducing apoptotic events in part of the ESC population. The remaining ESC population will be resistant to nitrosative stress and express the cytoprotective genes haeme oxygenase-1 and HSP70, representing the start of a differentiation programme^[18].

Mitochondria and stemness

Mitochondrial modulation is emerging as a mediator of stem cell proliferation and differentiation. Mitochondrial function is known to be fundamental to cellular health. The two actions that maintain mitochondrial function are fission and fusion processes, collectively termed mitochondrial dynamics (MD). Altering the balance of MD results in changes to mitochondrial morphology and increases the incidence of age-related disorders, such as neuromuscular degeneration, and of metabolic disorders, such as obesity, impaired glucose tolerance, and diabetes^[33,34]. Many of these disorders have been shown to originate due to alterations in the function, morphology and number of mitochondria. The volume and efficacy of the mitochondrial mass is considered a determining factor in the production of reactive oxygen species (ROS) and the response to the oxidative stress level^[35]. It has been reported that ROS levels are lower in undifferentiated cells. ESCs have been reported to resist oxidative stress better than differentiated cells and to contain a large complement of active mitochondria^[36]. In addition, it has been shown that the expression levels of pluripotency markers are downregulated in mESCs when the mitochondrial DNA copy number is increased^[37]. In general, pluripotent stem cells (PSCs) have a low mitochondrial population with low energy potential; most of the energy comes from glycolysis, which is limited only by a low ATP reservoir that precludes glucose phosphorylation to glucose 6-phosphate, which is required for uptake into the cells. Several types of differentiated cells, such as those that have differentiated into the trophectoderm of mice and rats, have been described as having more

elongated mitochondria, with higher membrane potential and more O₂ consumption^[38]. When cells are differentiated, the number of mitochondria is observed to increase, and the mitochondrial morphology shows characteristics observed in mature cells^[39-41]. This behaviour is similar to that described in tumour cells, which have a decreased respiratory rate associated with an enhancement of anaerobic glycolysis due to a uniform transcriptional reduction of mitochondrial components. Human ESCs (hESCs) have been reported to have only a few mitochondria with immature morphology, and it has been found that the mitochondrial mass, the intracellular ROS level and the expression of antioxidant enzymes increase with differentiation^[41]. Thus, the ROS produced in the differentiated cells might play an important role in cell signalling and differentiation^[41-43]. Dynamic changes in mitochondrial energy metabolism, ROS and antioxidant enzymes have been shown to affect differentiation propensity. In fact, mitochondrial biogenesis is controlled by the expression of oxidative metabolism genes, among which are mitochondrial transcription factor A (Tfam), nuclear respiratory factor (NRF-1) and peroxisome proliferator activated receptor γ co-activator 1 α (PGC-1 α)^[44,45], which has been described to regulate mtDNA transcription and replication.

NO has been described as a physiological regulator of the mitochondrial respiratory chain and has been reported to interact with oxygen bound to CcO located in the inner mitochondrial membrane. CcO has a higher affinity for NO than for oxygen, which suggests that this interaction and its biological consequences are dependent on the redox state and turnover of CcO^[4,46]. It has been shown that NO maintains normal cellular ATP levels by inhibiting mitochondrial respiration and increasing glycolysis^[28]. This activity of NO is an important mechanism by which NO can modulate cellular responses to hypoxia in mammalian cells.

NO AND MITOCHONDRIAL BIOGENESIS

Mitochondrial biogenesis and mitochondrial dynamics

Mitochondrial biogenesis (MB) can be defined as the growth and division of pre-existing mitochondria as a mechanism to adjust the cellular energy balance in response to an environmental change or a change in the general status^[47,48]. This process is regulated by a wide range of substances, including benzodiazepine, Ca²⁺ fluxes, and thyroid hormones such as T3, which controls metabolic rates in vertebrates^[49,50]. Growing evidence suggests that the delicate equilibrium between mitochondrial fission and fusion is vital for many mitochondrial functions, including metabolism, energy production, Ca²⁺ signalling, ROS production and apoptosis^[49,51-53]. For example, in some neurodegenerative diseases, there is a reduction in the expression levels of fusion proteins such as optic atrophy type 1, mitofusin-1 and mitofusin-2 and an increase in the expression of fission proteins such as dynamin-related protein-1 and fission related protein-1^[54]. It has been reported that mitochondrial

to mitochondrial fragmentation and cell death. This result has also been supported by the activation of Mtn2 expression^[64]. In contrast, while PGC-1 α permits the tolerance of a certain level of toxins in the cell, the prolonged expression to non-physiological levels of PGC-1 α has a negative effect on mitochondrial function and the viability of the cells^[65]. This suggests that the maintenance of a homeostatic PGC-1 α expression level may offer a promising strategy for neuroprotective therapies against some toxicants^[60].

NITRIC OXIDE REGULATES MITOCHONDRIAL FUNCTION

NO plays a very important role in regulating mitochondrial function and cell metabolic activity. It has been described that endothelial nitric oxide synthase (eNOS) is associated with the outer mitochondrial membrane in neurons and endothelial cells, which suggests that NOS regulates mitochondrial function^[66] (Figure 1). The chemical structure of NO allows the interaction with haemoglobin and the release of O₂ for mitochondrial consumption^[67]. As introduced earlier in this review, a mechanism for the regulation of mitochondrial function is the binding of NO to CcO, the terminal enzyme in the electron transport chain^[68]. It competes with O₂ as the last electron receiver, inhibiting the activity of the enzyme and preventing water formation and ROS generation^[69,70]. Furthermore, at a low concentration of O₂, both physiological and low concentrations of NO inhibit CcO and induce a switch to glycolysis that permits adaptation to hypoxic conditions^[28,71]. However, a high concentration of NO also inhibits other mitochondrial complexes of the respiratory chain (complexes I, complexes II and complexes III), increasing superoxide anion (O₂⁻) production and inducing cell death^[70,72] (Figure 1).

The hypoxia response is mediated by hypoxia inducible factor (HIF). HIF1 α is the isoform that regulates oxygen homeostasis and cell metabolism in the short-term hypoxia response. Both HIF1 α and NO help to restore energy metabolism at a low oxygen concentration^[73]. High NO has been described to induce HIF1 α expression under normoxic conditions in a mitochondria-dependent manner, but the effect of low NO under normoxia is unknown^[27]. It is very important for us to evaluate this effect because we have evidence that low NO under normoxia induces HIF1 α and can activate a similar hypoxia response.

NO and cell metabolism in pluripotent stem cells

The increase in anaerobic respiration and the decrease in oxidative phosphorylation in the presence of available oxygen is known as the Warburg effect^[74]. This effect was considered a particular feature of cancer cells due to the typical hypoxic environment of tumours, but currently, it is considered a metabolic shift that permits cells to divide and proliferate^[75]. Because of this, reduction of

the ROS levels by the reduction of oxidative phosphorylation permits the activity of proliferative kinases, such as ERK1/2 and Akt, which inhibits the activation of the apoptotic machinery *via* activation of anti-apoptotic control mechanisms and the non-activation of pro-apoptotic kinases, such as c-Jun-NH₂-terminal kinase and p38 mitogen-activated protein kinase^[76].

It has been reported that somatic cells require a shift from oxidative to glycolytic metabolism for the reprogramming process. Both HIF1 α and HIF2 α are necessary in the early state of the reprogramming for this metabolic change and for the recovery of the pluripotent state^[77]. The bioenergetics of pluripotent cells can vary depending on their developmental stage. hECSs present highly glycolytic metabolism and share this feature with cancer cells (Warburg effect)^[78]. Because the stem cell niche presents an hypoxic environment, the glycolytic metabolism of undifferentiated cells could be an adaptation to low oxygen concentrations *in vivo*^[79]. In addition, the efficiency of the reprogramming process is reduced by glycolysis inhibition and an increase in glycolytic potency during the generation of inducible PSCs (iPSCs)^[80]. Furthermore, it has been described that hypoxia enhances the generation of iPSCs. HIF1 α and HIF2 α are essential for the metabolic changes required for early iPSC generation in humans. However, HIF2 α is detrimental at later stages of reprogramming because of the upregulation of TNF-related apoptosis-inducing ligand^[77].

Cell metabolism is remarkably important for determining the stem cell fate and the role of NO in the regulation of metabolism. Almeida *et al.*^[28] described that NO activates glycolysis in astrocytes *via* the phosphorylation of AMP-activated protein kinase (AMPK), which activates Phosphofructokinase 2 and protects cells from apoptosis.

The relationship between NO and cell metabolism could be vital for the expansion of pluripotent cells when NO is used as a supplement in the design of culture medium, and it seems reasonable that NO may be used as a pluripotency inducer (Figure 1).

HIGH NO INDUCES ERS

The endoplasmic reticulum (ER) is the organelle designated for the synthesis and folding of proteins that are directed for secretion or to the Golgi apparatus. Because proper protein synthesis and protein folding are the key functions of the ER, the interruption of this physiological process ends in a complex ERS response, with a goal of recovering physiological function. The ER also functions as a store for Ca²⁺ and regulates its homeostasis through Ca²⁺-pumping and Ca²⁺-releasing proteins located in its membrane. Ca²⁺ is also an essential ion for ER function. Many chaperones, such as calreticulin or protein disulphide isomerase, are dependent on Ca²⁺ concentration, and therefore, any variation in ER intra-organelle Ca²⁺ concentration leads to changes in ER function^[81,82].

There are many states that can lead to an unfolded protein response (UPR), the primary role of which is

to recover internal homeostasis and adapt to the new conditions in the ER. Any alterations in Ca^{2+} homeostasis can involve the UPR due to the malfunctioning of proteins responsible for protein folding in the ER lumen; nevertheless, the possible causes of the UPR are not restricted to this process, as, for example, glucose deprivation can also lead to ERS^[83].

The first stage in the UPR implies an attempt at adaptation triggered by the release of chaperones anchored in membrane proteins, mainly glucose regulate protein 78 (Grp78, also known as BiP), to prevent the accumulation of misfolded proteins in the ER lumen. This release of Grp78 entails the aggregation of membrane proteins to which Grp78 was attached as well as Grp78 auto phosphorylation. Among these proteins, we primarily found protein kinase-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (Ire-1) and transcription factor 6 (ATF6)^[84] (Figure 1).

PERK is a protein kinase that is able to auto phosphorylate when it oligomerizes, activating its kinase domain and inactivating eukaryotic initiation factor 2 α (eIF2 α) after its phosphorylation. This leads to a decrease of mRNA translation with the aim of reducing the protein mass in the ER. In turn, genes related with the UPR are preferentially translated, as is the case of the transcription factor ATF4^[85].

ATF4 is a CHOP activator and an apoptotic enhancer^[86]. CHOP has been involved in many processes that lead to cellular apoptosis through the regulation of genes related with the cell cycle and ROS generation^[87]. CHOP dimerizes with C/EBP, preventing its binding to gene promoters and blocking genes such as Bcl2, apoptotic suppressor, or peroxisome proliferator-activated receptor gamma, which promotes cell proliferation. This uniformly activates the expression of genes such as IL-6 and GADD34, which promotes cell differentiation and ROS synthesis^[82,86].

ATF6 is a transcription factor that acts in the ERS response. ATF6 is cleaved when unfolded proteins are accumulated in the ER, releasing its cytoplasmic domain, which translocates to the nucleus. The inactive form of ATF6 (p90ATF6) is transported to the Golgi and is activated via two-step cleavage by Site-1 protease and Site-2 protease. Then, the active form of ATF6 is transported to nucleus^[88] and functions as a transcriptional activator for ERS-related genes such as CHOP^[89] (Figure 1).

Ire1 is a protein that shares similar pathways with PERK. Upon its oligomerization after Grp78 is released, its kinase activity is activated, and Ire1 is able to process an intron from Xbp protein-1 (XBP-1) mRNA to trigger its translation. XBP-1 activates genes related to protein transport from the ER to the cytosol, resulting in its degradation^[82]. XBP-1 has also been correlated with apoptotic processes^[84] and lymphocyte differentiation^[83].

The release of Grp78 from the end of ATF6 also leads to its release towards the Golgi apparatus, where proteases are in charge of its cleavage, after which ATF6

is able to migrate to the nucleus and regulate genes, including the activation of XBP-1, which, as mentioned before, is an upregulator of CHOP^[85].

Despite previous research on the ERS response and the effects of NO in cell regulation, the connection between these two processes was not clarified until 2001, when the first relationships between NO and CHOP were established through Ca^{2+} release and the subsequent release of ER Ca^{2+} to the cytosol induced by NO. Increases in the cell NO concentration lead to Ca^{2+} release from the mitochondria due to Cyt c inhibition, and this unleashes Ca^{2+} release in the ER. This response to NO is triggered as a result of protein misfolding in the ER because many chaperones are Ca^{2+} -dependent, which causes a UPR that over time ends in an apoptotic response^[90-92] (Figure 1).

Endoplasmic reticulum stress and cell differentiation

High concentrations of NO have been frequently associated with differentiation and apoptotic processes; nevertheless, the genetic mechanisms that initiate these processes are barely defined^[93]. Recently, many reports have suggested the relevance of the ERS response in the differentiation of several cell lines, which suggests the possibility of studying this response as a possible differentiation mechanism triggered by high concentrations of NO (Figure 1).

Previous studies have demonstrated the importance of ERS for the differentiation of chondrocytes^[94-96], plasma cells^[95,97], adipocytes^[98] and myoblasts^[18]. Nonetheless, recently, the differentiating potential of ERS has been identified in many other cell lines. Saito *et al.*^[99] demonstrated the effect of ERS in the terminal differentiation of osteoblasts through the activation of the PERK-eIF2 α -ATF4 pathway. The results concluded that PERK activation triggered by the ERS response is required for ATF4 activation in osteoblast differentiation and for the proper expression of genes that are essential for osteogenesis, such as Ocn and Bsp^[99]. Heijmans *et al.*^[100] studied the loss of pluripotency in epithelial intestinal cells upon activation of the UPR. They proposed that the ERS response causes the loss of stemness in a manner dependent on Perk-eIF2 α -ATF4, a pathway activated in the UPR. Their findings demonstrate that inhibition of this signalling pathway results in stem cell accumulation in the epithelium, suggesting that the ER stress response is a key factor in the differentiation of this cell type^[101,102]. More recently, Matsuzaki *et al.*^[101] evaluated the effect of physiological ER stress in fibroblast differentiation, suggesting that fibroblasts subjected to RER are more susceptible to differentiation signals and implying that UPR signalling could be essential for this differentiation. This is significant, considering that these cells are subject to a high protein load in the ER lumen in their final differentiation state due to the high amount of protein directed to secretion^[96,100].

These studies suggest the possibility of studying the effect of NO in differentiation through this response

because the precise control of this molecule in this cellular process has been at least partially characterized.

Physiological concentrations of NO and RER

Although NO is a molecule that in high concentrations can lead to apoptosis and differentiation processes, some studies have suggested its protective effect and have demonstrated that endogenous levels of NO produced by different types of NOS are necessary for proper cell function and pluripotency maintenance. Previous studies have shown the need for physiological levels of NO to avoid the generation of free radicals or the activity of many proteases or to increase the antioxidant potential of GSH^[103].

In 2007, Kitiphongspattana *et al.*^[97] demonstrated that the constitutive production of NO by cNOS was essential for a proper ER response in β pancreatic cells. Its inhibition repressed the expression of genes involved in protein folding and antioxidant defence, such as Gclc, Grp78, Prx-1 or Gpx-1^[88,97]. Moreover, a protective effect of physiological NO in β -cells through the activation of Nrf2, a transcription factor for antioxidant proteins, in the RER has been reported previously, as has the importance of the UPR in resistance to oxidative stress^[104-106].

Hypoxia, ERS and AMPK protective effect

ERS activates apoptosis through several stimuli, including hypoxia, oxidative stress and Ca^{2+} depletion. AMPK activation has been shown to protect cardiomyocytes against hypoxic injury through the attenuation of ERS^[107].

ERS promotes apoptotic signalling or cell survival in different cell types. Therefore, the decision between survival and apoptosis may depend on the balance between survival signalling and apoptotic signalling.

Three apoptotic pathways are known to be related to ERS. Among others, the induction of the gene for CHOP promotes apoptosis, and CHOP deficiency can protect cells from ER stress-induced apoptosis, suggesting that CHOP is involved in the process of cell death caused by ERS. Other authors proposed that the mechanism of ERS induction during hypoxia is the alteration of the Ca^{2+} balance *via* the inhibition of the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase pump due to intracellular ATP depletion^[108]. Because of this, Terai *et al.*^[107] hypothesized that conservation of the intracellular ATP content during hypoxia would protect cardiomyocytes from ERS-induced cell death. They concluded that AMPK would be a cell protector against ERS-induced cell death during hypoxia because AMPK acts as an intracellular energy sensor, maintaining the energy balance within cells during ischaemia. The mechanism by which AMPK protects cells during hypoxia is attributed to the suppression of protein synthesis due to the phosphorylation of eEF2. This protective effect mediated by AMPK has also been described in another recent study. Hwang *et al.*^[109] reported that AMPK mediated cell survival induced by resveratrol in H9c2 cells, and this may exert a novel therapeutic effect on the oxidative stress induced in

cardiac disorders.

It is interesting to consider the effects of AMPK with regard to the role of NO in the activation of this kinase. NO has a protective role against ROS and ERS, and this action could be mediated by AMPK. As we have demonstrated, NO regulates cell metabolism, and this effect can help to maintain pluripotency. NO has become known as a potent molecule that regulates cell protection.

CONCLUSION

In this literature review, we would like to highlight the role of NO as a regulator of stem cell properties. Low NO concentrations have been shown to help to maintain pluripotency, but the molecular mechanisms of this effect are not yet known.

We have analysed the role of NO in mitochondrial function and ERS because this pathway is interrelated with stem cell fate and could be an explanation for the mechanism by which NO regulates embryonic development.

We have described that NO has an important role in mitochondrial biogenesis and the induction of PGC1 α expression. Notably, PGC1 α has a neuro-protective effect against certain levels of different toxins. Due to the relationship between NO and PGC1 α , the information in this review suggests the importance of studying the potential protective role of NO in cells.

NO is highly involved in the ERS response through its effect on mitochondria initiation of an ERS response, initially triggered by the release of Ca^{2+} in the mitochondria. This ERS response originates differentiation processes in many cell lines. This suggests the importance of studying the effect of NO in the ERS response to clarify its different effects through this pathway.

Likewise, NO in the ER is also relevant at physiological concentrations because many studies have shown that a proper ERS response is not feasible without sufficient physiological intracellular NO synthesis.

NO is involved in the regulation of cell respiration at a physiological level. NO inhibits cytochrome c and induces glycolysis, which can help to regulate stem cell fate. Pluripotent cells have been reported to have a highly glycolytic metabolism. In the reprogramming process, it is necessary that restoration of glycolytic metabolism is mediated by hypoxia-inducible factors. The induction of glycolysis by NO is vital for using this molecule as an inducer of pluripotency. NO is a strong tool for culturing pluripotent cell lines in an undifferentiated state. Moreover, NO can be used for biotechnology applications in the design of a culture medium for pluripotent cell expansion and for the creation of a cell therapy programme.

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Impact of T cells on hematopoietic stem and progenitor cell function: Good guys or bad guys?

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Abstract

When hematopoietic stem and progenitor cells (HSPC)

are harvested for transplantation, either from the bone marrow or from mobilized blood, the graft contains a significant number of T cells. It is these T cells that are the major drivers of graft-*vs*-host disease (GvHD). The risk for GvHD can simply be reduced by the removal of these T cells from the graft. However, this is not always desirable, as this procedure also decreases the engraftment of the transplanted HSPCs and, if applicable, a graft-*vs*-tumor effect. This poses an important conundrum in the field: T cells act as a double-edged sword upon allogeneic HSPC transplantation, as they support engraftment of HSPCs and provide anti-tumor activity, but can also cause GvHD. It has recently been suggested that T cells also enhance the engraftment of autologous HSPCs, thus supporting the notion that T cells and HSPCs have an important functional interaction that is highly beneficial, in particular during transplantation. The underlying reason on why and how T cells contribute to HSPC engraftment is still poorly understood. Therefore, we evaluate in this review the studies that have examined the role of T cells during HSPC transplantation and the possible mechanisms involved in their supporting function. Understanding the underlying cellular and molecular mechanisms can provide new insight into improving HSPC engraftment and thus lower the number of HSPCs required during transplantation. Moreover, it could provide new avenues to limit the development of severe GvHD, thus making HSPC transplantations more efficient and ultimately safer.

Key words: Hematopoietic stem cells; Hematopoietic stem and progenitor cells; CD8 T cells; Transplantation; Engraftment; Memory T cells; Facilitating cells; Bone marrow

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Core tip: T cells act as a double-edged sword upon allogeneic hematopoietic stem and progenitor cells (HSPC) transplantation, as they support engraftment of HSPCs and provide anti-tumor activity, but are also the cause

of graft-vs-host disease (GvHD). Here, we discuss the findings from several studies that have addressed the still enigmatic role of T cells during HSPC transplantation, either in an allogeneic or autologous setting, in mice or men, and with HSPCs derived from bone marrow, peripheral blood or cord blood. We anticipate that a better comprehension of how T cells support HSPC engraftment may lead to new strategies to optimize HSPC transplantations and prevent GvHD.

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INTRODUCTION

Over the past 60 years, hematopoietic stem cell transplantation (HSCT) has developed into routine treatment for several hematological and immunological malignancies and deficiencies. Hematopoietic stem cells (HSCs) are the rare, self-renewing progenitor cells at the top of the hematopoietic hierarchy that can give rise to all blood cell lineages, and are thus able to restore hematopoiesis and the immune system in transplanted recipients. Hematopoietic stem and progenitor cells (HSPCs) are a mixture of HSCs and more differentiated progenitor cells, which provide respectively long and short-term repopulation of blood cells following transplantation. In the past, HSPCs required for transplantation were solely acquired from the bone marrow (BM), typically from the hipbone. This is an invasive procedure and if the BM harvest was not optimal, this often resulted in reduced engraftment of HSPCs and limited hematopoietic/immunologic recovery. Transplantation of higher doses resolved the majority of engraftment problems. Nowadays, this is facilitated by more favorable and less invasive HSPC harvesting procedures from mobilized peripheral blood (MPB) or umbilical cord blood (UCB). In the case of MPB HSPCs, donors are usually pretreated with granulocyte colony-stimulating factor (G-CSF), which mobilizes the HSPCs from the BM into the blood stream^[1,2]. Subsequently, donors undergo one or two apheresis sessions to reach the desired HSPC dose for transplantation, normally $2-10 \times 10^6$ CD34⁺ cells/kg recipient body weight^[3-5]. In case of UCB HSPCs, the UCB is collected from the postpartum placenta, as this is a rich source of HSPCs. Typically, two grafts are required to reach sufficient numbers of UCB HSPCs when transplanting an adult. UCB HSPC transplants are generally used in an allogeneic transplantation setting, whereas mobilized HSPCs are used both during autologous and allogeneic HSCT. Autologous HSCT following high-dose chemotherapy has become routine treatment for many types of lymphomas^[6]. It is considered a relatively low risk treatment, as the recipients receive their own HSPCs that were harvested before the chemotherapy

was started. Allogeneic HSCT is regarded as a more precarious procedure, as HSPCs from a non-self origin are transplanted, which are thus subject to rejection by the host. In addition, allogeneic HSCT is combined with conditioning regimes of different intensity, leading to more variable transplantation outcomes. As with any allogeneic transplantation, matching of the human leukocyte antigen (HLA)-profile between donor and patient is instrumental to lower the risks of graft rejection. The first step in finding a suitable donor is to search within the immediate family. Related siblings have a 25% chance of being perfect donors, *i.e.*, matching 10 out of the 10 HLA antigens. Unrelated donors can also match the HLA antigens perfectly, although the chance of finding a perfectly HLA-matched, unrelated donor in a timely manner is much lower. Transplantation with partially matched (haploidentical), related donors (parent, child or sibling) is now considered as a viable alternative. Although these donors are usually more readily available, haploidentical donors have more HLA-disparities when compared to the recipient. This increases the risk of graft rejection, but also the development of graft-vs-host disease (GvHD)^[7]. GvHD is a complicating side effect of an allogeneic HSPC transplantation that can develop early or late after the transplantation, in which transplanted donor T cells mount an extensive immune response against the recipient's tissues. A mild degree of GvHD is considered beneficial when the HSPC transplantation is part of an anti-cancer treatment, as the allo-responsive donor T cells in the graft can also eradicate the remaining tumor cells present in the recipient. However, severe forms of GvHD are very hazardous and hence the predominant cause of the high morbidity and mortality rate associated with allogeneic HSPC transplantations. A highly efficient procedure to minimize the risk of developing acute and chronic GvHD is T cell depletion (TCD) from donor grafts^[8]. Unfortunately, transplantations with TCD grafts also revealed major pitfalls, such as graft failure and disease relapse. This was observed in BM transplants with grafts from HLA-identical siblings and HLA-non-identical (but related) donors with minimal mismatch^[8,9]. What was apparent from these studies is that conditioning regimes and post-transplant immune suppression treatments can all affect the outcome of transplantation with TCD grafts. To this day, it is still unclear how the depletion of T cells leads to increased risk of graft failure. What is certain is that T cells can aid the engraftment of HSPCs in the damaged BM environment in which they find themselves post-transplant. Here, we review what is currently known about the contribution of T cells on HSPC engraftment in different transplantation settings, as this knowledge can be used to improve both the efficiency and safety of this important clinical procedure.

FACILITATING T CELLS

While grafts depleted of T cells minimize the risk of developing GvHD, T cell depletion also compromises the engraftment of transplanted HSPCs. This unwanted effect

has been attributed to the loss of specific cell subsets that assist the engraftment, the so-called facilitating cells (FC) that were depleted during the T cell depletion process. In 1994, Kaufman *et al.*^[10] showed for the first time that engraftment of murine BM HSPCs was facilitated by BM cells that were positive for CD8, CD45, CD45R, CD3, dull/intermediate for MHC class II and negative for the TCR. The absence of the TCR indicates that these cells are not T cells. However, some discrepancy does exist regarding this finding. Gandy *et al.*^[11] observed increased survival of mice transplanted with allogeneic HSPCs together with BM CD8⁺ cells. When separated into TCRβ⁺ and TCRβ⁻ fractions, both CD8⁺ TCRβ⁺ and CD8⁺ TCRβ⁻ cells had the ability to enhance survival of transplanted mice. The TCRβ⁺ population displayed morphology resembling T lymphocytes. The majority of the TCRβ⁻ cells had granular cytoplasm and low nuclear to cytoplasm ratio with a lymphoid dendritic cell phenotype. Further examination on the CD8⁺ TCRβ⁺ cells, which are predominately present in the BM, revealed that they express CD44^[11]. This indicates that these T cells have a memory phenotype. In the BM, three different subsets of CD8⁺ T cells co-exist; effector memory (T_{EM}; CD44⁺ CD62L⁻), central memory (T_{CM}; CD44⁺ CD62L⁺) and naïve T cells (T_{NV}; CD44⁻ CD62L⁺)^[12]. We recently showed that the frequency of total CD8⁺ T cells is similar within the different bones found throughout the murine body. Additionally, we observed that during steady state conditions, T_{NV} cells are the dominant subset and that this quickly changes after an acute infection with lymphocytic choriomeningitis virus, as the T_{EM} cells replace the T_{NV} cells^[13]. Also in humans, it has been shown that CD8⁺ cells are important for HSPC engraftment. Martin *et al.*^[14] demonstrated that removal of CD8⁺ cells but not CD4⁺ cells from donor bone marrow grafts results in graft failure. Interestingly, they also showed that the dose of the CD8⁺ cells in the grafts is of major importance as more than half of the patients that received grafts containing less than 3.9×10^6 CD8⁺ cells/kg experienced graft failure. They further observed that the high dose of CD8⁺ cells required to prevent graft rejection also increases the risk for acute and chronic GvHD^[14]. Taken together, many studies in mice and human have quite clearly shown that CD8⁺ T cells have a beneficial effect during HSCT. This in itself is surprising given the fact that T cells, depending on their activation state, are known to have a strong skewing impact on hematopoiesis. We and many others have shown that activated T cells can directly affect HSC function by increasing differentiation and limiting self-renewal^[15]. Interferon-gamma (IFN-γ) is one of the pro-inflammatory cytokines produced by activated T cells, which can inhibit HSC self-renewal and enhance their differentiation in a direct manner^[16,17], but also indirectly by acting on surrounding niche cells^[18,19]. This indicates that the impact that T cells can have on the behavior and function of HSPCs is complex and not only dependent on the T cell subset, but also on the activation status of the T cells and the niche cells they interact with.

THE INFLUENCE OF T CELLS ON HSPC ENGRAFTMENT DURING ALLOGENEIC AND AUTOLOGOUS TRANSPLANTATION

Although early studies demonstrated a clear contribution of CD8⁺ T cells on HSPC engraftment, this concept received surprisingly limited follow-up, both scientifically and clinically. This is most likely due to the fact that current transfusion protocols are performed with high numbers of HSPCs, which compensates for any sub-optimal engraftment condition^[14]. Nonetheless, understanding how to optimize the engraftment potential of HSPCs is still desirable, especially when HSPC numbers are limited. We therefore also took into account studies that investigated which T cell subsets are involved in the development of GvHD. The majority of these studies did not examine HSPC engraftment *per se*, but do provide interesting clues on which T cell subsets may be beneficial for this process, as they did assess immune reconstitution after transplantation. Chen *et al.*^[20] depleted CD62L⁺ T cells (T_{NV} and T_{CM}) from murine BM grafts and found that CD62L⁻ T cells (T_{EM}) accelerated the recovery of CD4⁺ and CD8⁺ T cells after transplantation, which could indicate enhanced hematopoietic engraftment. In addition, transplantation of the T_{EM} subset alone did not result in GvHD, while grafts that still included T_{NV} and T_{CM} did. Another interesting observation made is that transplantation of CD62L⁻ T cells led to increased donor chimerism, as it lowered the numbers of residual recipient T cells^[20]. Similar results were obtained in human studies. Naïve human T cells express CD45RA and switch to the CD45RO isoform upon antigen encounter and develop into memory T cells. Touzot *et al.*^[21] demonstrated that allogeneic HLA-mismatched HSCT with CD45RA-depleted BM grafts were successful and did not lead to severe GvHD, suggesting that this could be a potential approach to treat patients with primary combined immuno-deficiencies. Importantly, they also observed that viruses detected prior to the HSCT were rapidly cleared post-HSCT, indicating that the CD45RA-depleted graft contained CD8⁺ T cells functionally active against pathogens. Furthermore, Triplett *et al.*^[22] found similar results when transplanting patients suffering from hematological malignancy with haploidentical CD45RA-depleted grafts. Here, patients received on day 0 G-CSF MPB grafts that was highly enriched for CD34⁺ cells and thus depleted of CD3⁺ cells, with a median dose of 11.2 and 0.012×10^6 /kg, respectively). On day 1 patients received MPB grafts depleted of CD45RA⁺ T cells (and thus enriched for memory T cells), and on day 6, they received an infusion with purified NK cells from the same donor^[22]. The authors demonstrated that this combination of differently prepared grafts led to rapid neutrophil engraftment, quick conversion to full donor chimerism and fast reconstitution of innate and adaptive immunity. These three clinical parameters signify rapid HSPC engraftment, which may well have been the result of the co-injected CD45RO⁺ memory T cells on day 1, although this was not formally tested in this study.

Importantly, none of the patients developed acute GvHD. Several patients did show signs of chronic oral GvHD, but not of severe nature^[22]. Next to removing specific subsets that might cause GvHD, many studies also focused on adding back T cells after transplantation. The transfer of donor T cells after transplantation has in fact become a frequently applied clinical procedure, called donor lymphocyte infusion (DLI). This is usually performed to cause a milder and more controllable degree of GvHD, aiming for the donor T cells to eliminate residual recipient cells and thereby improve donor chimerism and/or remove residual tumor cells (graft-vs-tumor effect). For DLI, peripheral blood is donated by the same HSPC-donor, this time without G-CSF pre-treatment. Next, CD3⁺ cells are isolated and given to the patient after a brief recovery period after the initial HSCT^[23]. A study performed in mice found that DLI was able to improve engraftment of HSC without resulting in GvHD^[24]. They observed that mice, which had received low (sub-lethal) dose of irradiation, rejected allogeneic donor BM cells, unless this procedure was combined with an injection of peripheral blood mononuclear cells. Further analysis revealed that CD8⁺ cells were facilitating the engraftment. Importantly, this beneficial effect was only observed when the DLI was given on the same or the following day, but not on third or seventh day of the HSCT^[24]. This suggests that early DLI may be sufficient to counteract the beginning of an anti-donor response by boosting HSPC engraftment. Interestingly, a study in humans did not show similar effect of DLI; Kreiter *et al.*^[25] found that minimal conditioning prior to T cell-reduced allogeneic HSCT combined with subsequent DLI was insufficient to sustain long-term engraftment. This study, similar to Nakamura's study, gave DLI on the same day as the HSCT. However, the fact that none of the transplanted subjects reached complete donor T cell chimerism indicates that this specific combination of minimal conditioning and DLI dose was not optimal^[25]. In contrast to the mice in Nakamura's study, these human subjects were patients suffering from hematological malignancies. It remains to be determined if and how the conditioning regimes and the presence of disease affect the possible facilitating role of DLI on HSPC engraftment. Interestingly, also in a DLI setting, infusion of T cells depleted of naïve T cells was shown to favor engraftment without causing GvHD^[26]. Additional investigation on the murine CD62L⁻ T cell subset originally described by Chen *et al.*^[20] revealed that addition of 1×10^6 CD62L⁻ T cells could rescue 90% of graft rejection that developed after transplantation with TCD grafts in mice. The CD62L⁻ T cell subset was also shown to prevent tumor growth and help combat viral infection^[26]. More importantly, addition of CD62L⁻ T cells resulted in 100% donor chimerism within 30 d and was maintained long-term. Similar results were obtained in human studies, as Shook *et al.*^[27] transplanted patients with CD3⁺-depleted haploidentical grafts and infused CD45RA-depleted cells the following day. They

observed that all patients reached complete donor chimerism. In this study patients received myeloablative conditioning. Remarkably, 3 mo after the HSCT, the majority of CD4⁺ and CD8⁺ T cells were still CD45RA⁻, indicating that the T cells were derived from the CD45RA-depleted grafts^[27]. DLIs can also be given to patients not responding to anti-viral medication during an infection post HSCT. This is particularly important after HSCT with T cell-depleted grafts, as protective immunity is not transferred in this setting and engraftment, and thus immune reconstitution is delayed. Stemberger *et al.*^[28] demonstrated that as few as 3750 antigen-specific T cells per kg body weight was sufficient to decrease viral load in a patient suffering from systemic CMV infection after CD3-depleted HSCT^[28]. In this study, the impact on HSPC engraftment was not reported. Nevertheless, this study highlights a feasible strategy to combat persisting infections post HSCT. So far, the facilitating role of CD8⁺ T cells, especially memory T cells in HSPC engraftment has been well established in allogeneic HSCT setting. Less explored is if these cells have similar effects in autologous HSCT. Interestingly, Rutella *et al.*^[29] demonstrated that patients undergoing autologous transplantation with selected CD34⁺ cells instead of unmanipulated PBMCs experienced delayed repopulation of the T cell lineage. This suggests that the lack of T cells might also affect engraftment in an autologous setting. Furthermore, Russell *et al.*^[30] assessed mobilization and engraftment in autologous donors. They observed that grafts with low numbers of CD34⁺ cells (poor mobilizers) contain more CD8⁺ T cells than grafts from moderate or high mobilizers. Subsequently, they assessed if the CD8⁺ T cell content was associated with time to neutrophil engraftment after HSPCT. The results obtained suggest that engraftment occurs faster when there are more CD8⁺ T cells present in the grafts. This was limited to grafts that contain low numbers of CD34⁺ cells^[30]. Taken together, these studies show that when HSPC numbers are limiting, memory CD8⁺ T cells have a beneficial effect on HSPC engraftment, both in allogeneic and autologous transplantation. The effects of different (graft) treatments on HSCT are summarized in Table 1.

THE ROLE OF T CELLS UPON TRANSPLANTATION OF HSPCS FROM UMBILICAL CORD BLOOD

In the clinic, when an HLA-matched sibling and unrelated matched donor are not available, the next best option is HSCT with UCB. It is now well established that transplantation with UCB grafts results in lower rates of GvHD disease when compared to HSCT from BM or MPB. This in itself is very interesting, especially as the majority of the T cells present in a UCB graft are CD45RA⁺^[31], and thus potentially capable of inducing GvHD. However, these CD45RA⁺ UCB T cells are functionally distinct and less mature than CD45RA⁺ naïve T cells found in

Table 1 Effects of (graft) treatments on hematopoietic stem cell transplantation

Treatment	Removed	Remaining	Effect
Complete T cell depletion	All T cells	N/A	GvHD ↓ ^[8,14,44] Disease relapse ↑ ^[8,9,44] Graft failure ↑ ^[8,9,44] Immune reconstitution ↑ ^[44]
Partial T cell depletion	CD45RA (T _{NV}) CD62L ⁺ (T _{NV} , T _{CM}) ¹	CD45RO (T _{MEM}) CD62L ⁺ (T _{EM}) ¹	GvHD ↓ ^[20,21,22] Neutrophil engraftment ↑ ^[22] Immune reconstitution ↑ ^[20] Protective immunity ↑ ^[21,22] Donor chimerism ↑ ^[20,22]
Donor lymphocyte infusion	CD45RA (T _{NV}) CD62L ⁺ (T _{NV} , T _{CM}) ¹	CD45RO (T _{MEM}) CD62L ⁺ (T _{EM}) ¹	GvHD ↓ ^[26] Tumor growth ↓ ^[26] Engraftment ↑ ^[26] Graft failure ↓ ^[26] Immune reconstitution ↑ ^[26] Protective immunity ↑ ^[26,28] Donor chimerism ↑ ^[26,27]

¹Signifies the murine equivalent of the human T cell subset described above. Here, we summarize the impact that either full or partial T cell depletion of an HSPC graft, or selective donor lymphocyte infusion, can have on the clinical outcome of a HSPC transplantation. Indicated are the T cell subsets that have either been removed or that remain, and the biological or clinical effects that have been reported following this treatment. N/A: Not available; HSPC: Hematopoietic stem and progenitor cell; GvHD: Graft-*vs*-host disease.

adults^[32], explaining why these cells are less related to the development of GvHD. T cells in UCB have the ability to respond to allogeneic stimulation, but the response generated is less cytotoxic than that of adult T cells. Additionally, dendritic cells present in UCB have been found to be in an immature state and thus limiting the activation of UCB T cells^[32]. Nevertheless, while the development of GvHD is reduced, patients who undergo UCBT are subjected to high incidence of infection, as immune reconstitution is slow. It is believed that the low numbers of HSPCs and downstream progenitors transplanted during UCBT compared to MPB HSCT are the cause of the delay in hematopoietic reconstitution, while the absence of memory T cells would render the recipient more sensitive to viral infections. However, following our line of reasoning, the lack of memory T cells during UCBT might contribute to impaired HSPC engraftment. Currently, the focus on improving engraftment has been on reducing conditioning regimes, performing double UCB transplantation, *ex vivo* expansion of UCB HSPCs and intra-bone infusion of UCB grafts^[31]. An interesting approach is the combination of UCB grafts with CD34⁺ cells isolated from haploidentical grafts^[33]. The idea behind this concept is that the haploidentical graft will provide early engraftment, while the UCB graft provides long-term engraftment. Indeed, the authors observed fast engraftment of neutrophils and platelets post haplo-cord SCT. Furthermore, UCB cells replaced this first wave of hematopoiesis by the haploidentical CD34⁺ cells within 100 d. An important future aspect of this approach is that not the cell number of the UCB graft but rather the matching of the HLA type to that of the patient will take priority when finding suitable UCB grafts for UCBT. This by itself will provide more options for adult patients lacking related and unrelated HLA matched donors.

POSSIBLE UNDERLYING MECHANISMS ON HOW CD8⁺ T CELLS IMPROVE ENGRAFTMENT OF HSPCS

The underlying reasons on why and how CD8⁺ T cells contribute to HSPC engraftment are still poorly understood. Currently, there are more questions than answers, which we will address here; the mechanisms discussed below are depicted in Figure 1. For example, do donor T cells contribute to engraftment by killing residual host HSPC and thus eliminating the competition? This is unlikely, as Gandy *et al.*^[11] showed that CD8⁺ T cells did not facilitate HSPC engraftment *via* their lytic potential, as CD8⁺ T cells deficient in their lytic activity were still able to assist engraftment. However, one of the most important observations made by several studies is that the addition of donor T cells eliminates residual host T cells in mice^[20,24,26]. These observations suggest that removal of residual host T cells is an essential part in eliminating any type of resistance from the host to allow engraftment. Furthermore, is it possible that donor T cells somehow directly affect the function of HSPCs? An interesting observation made by Adams and colleagues is that in the $\beta 2m^{-/-}$ NOD/SCID mice, CD8⁺ cells augmented homing and engraftment of CD34⁺ cells by modulating their response towards CXCL12 by affecting their phosphotyrosine-mediated signaling. *Ex vivo*, this modulated response towards CXCL12 resulted in increased migration through a BM endothelial cell line^[34]. Further analysis revealed that this was not the result of factors secreted by CD8⁺ cells, though an active cytoskeleton in the CD8⁺ cells was required for the increased transmigration of CD34⁺ cells^[34]. Moreover, it could also be that CD8⁺ T cells can affect the HSC niche by making the environment more favorable for engraftment of the newly arrived HSPCs.

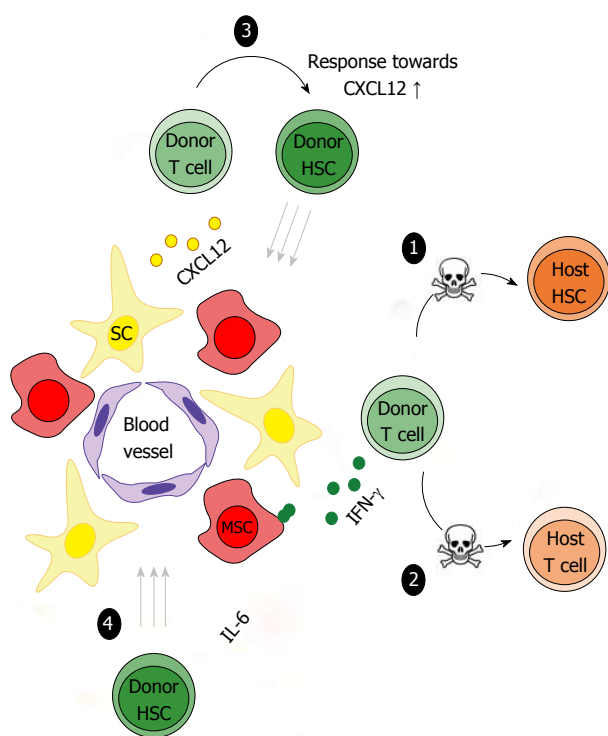


Figure 1 Potential mechanisms on how CD8⁺ T cells improve hematopoietic stem and progenitor cells engraftment. The following modes of action have been described or suggested by which donor T cells can support the engraftment of HSPCs upon transplantation: (1) killing of residual host HSCs; (2) killing of residual host T cells; (3) augmented homing of HSPCs to CXCL12 produced by reticular SC or MSCs; (4) increased HSPC differentiation by IFN- γ -induced production of IL-6 by MSCs. HSPCs: Hematopoietic stem and progenitor cells; SC: Stromal cells; MSCs: Mesenchymal stromal cells; IFN- γ : Interferon-gamma; IL-6: Interleukin-6.

The production of IFN- γ by CD8⁺ T cells was shown to promote the release of interleukin-6 from mesenchymal stromal cells (MSCs), an essential component of the HSC niche^[18]. This enhanced myeloid differentiation of more committed progenitors, though the impact of T cell-modulated MSCs on HSPC engraftment has not been examined. Lastly, an intriguing question on the functional impact of CD8⁺ T cells on HSCT is whether CD8⁺ T cells and HSCs co-localize in the BM. It has been shown that memory CD8⁺ T cells co-localize with VCAM-1-expressing stromal cells in BM^[35], whereas HSC-supporting MSCs also express VCAM-1^[36,37]. These findings are compatible with the hypothesis that HSCs and CD8⁺ memory T cells share the same niche, though actual co-localization between these cells has not yet been experimentally demonstrated.

THE IMPACT OF OTHER FACILITATING CELL TYPES ON HSPC ENGRAFTMENT

Although the positive impact of CD8⁺ T cells on HSPC engraftment has been addressed most extensively, there is evidence that other cell types in the BM can also have this effect. As mentioned before, not all CD8⁺ cells that can facilitate HSPC engraftment also express the TCR^[10]. In follow-up of these findings, Grimes *et*

al^[38] found that CD8⁺TCR⁻ FC do not express TCR gene transcripts (TCR α and TCR β), clearly distinguishing them from conventional T cells. Furthermore, they showed that CD8⁺TCR⁻ FC do express CD3 ϵ and that this complex is important for the beneficial effect of these cells during allogeneic transplantation^[38]. Further gene expression analysis on the CD8⁺TCR⁻ FC revealed that the DOCK2 gene was most significantly different between functional and functionally impaired FC cells. Indeed, FCs lacking the expression of DOCK2 do not enhance engraftment and do not promote homing and lodgment of HSPCs in the bone marrow^[39]. Additionally, also human CD3⁺CD8⁺TCR⁻ cells have been shown to have facilitating potential when co-transplanted with suboptimal doses ($3-5 \times 10^4$) of UCB CD34⁺ cells in NOD/SCID mice^[40].

Next to CD8⁺ T cells, multiple studies have shown that CD4⁺ T cells, especially regulatory T cells (Tregs) can also support HSPC engraftment. Danby and colleagues showed that higher proportions of Tregs in MPB grafts improve recovery and clinical outcomes^[41]. It has also been demonstrated that host Tregs co-localize with transplanted allo-HSPCs in BM^[42], indicating that these cells may provide an immune privilege site for HSPCs in the BM. Furthermore, also TCR $\gamma\delta$ ⁺ T cells have gained recognition for their facilitating role in engraftment of HSPCs. Kawanishi *et al*^[43] found that engraftment was associated with the dose of TCR $\gamma\delta$ ⁺ T cells present in BM grafts. Importantly, they found no association between the TCR $\gamma\delta$ ⁺ T cells dose and an increased risk for the development of acute GvHD in patients that received grafts from related donors^[43]. In conclusion, it is clear that the BM contains multiple cell types that can enhance HSPC engraftment. This is highly relevant from a clinical perspective, though it remains unclear to what extent these cell types also support the function or maintenance of HSPCs in the BM under physiological conditions.

FUTURE PERSPECTIVES

After decades of development in the allogeneic HSCT field, GvHD is still a major complication. To this day, the best approach for decreasing the risk for GvHD is the removal of T cells from the graft. After it was apparent that TCD procedure led to poor engraftment, delayed immune reconstitution and increased disease relapse^[44], TCD procedures were dismayed as a reliable method to safely and efficiently combat GvHD. However, several studies discussed here suggest that future of TCD HSCT may lie in partial instead of complete depletion of T cells. The aim of the variety of the approaches attempted so far was to remove T cells that contribute to GvHD while maintaining T cells that provide immediate but also long-term immune protection. The focus has never necessarily been to improve engraftment of HSPCs, as this potential problem is covered by the immense amounts of HSPCs transplanted. Nevertheless, identifying T cell subsets that specifically favor HSPC engraftment and the underlying mechanism may be more beneficial in the long run, as more efficient and rapid engraftment will be required if

less and less intense conditioning regimes are used in the future. Moreover, efficiency of HSPC transplantation is significantly reduced when donor HSPCs numbers are limited or when HSPCs are genetically modified, which thus requires protocols in which their engraftment is fully optimized. More in depth studies are required to determine whether the future of allogeneic HSCT can/will develop into one where patients receive minimal conditioning with a low dose of HSPCs followed by multiple infusions of different T cells subsets; these could be chosen based on their ability to improve HSPC engraftment, to provide the first wave of protective immunity and/or to induce a low-grade level of GvHD to boost an anti-tumor response. Unpublished work from our group suggests that *ex vivo* expanded CD8⁺ T cells with a memory phenotype have facilitating potential at the level of HSPC engraftment. It is hence interesting to speculate that the future of transplantation may include the addition of *ex vivo* expanded T cells from the graft in order to enhance engraftment and immune reconstitution. Hopefully, the developments in HSCT treatment in the coming years will result in faster recovery, decrease disease relapse and overall shorter hospital stays.

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- 45 Immunomodulatory oligonucleotide IMT504: Effects on mesenchymal stem cells as a first-in-class immunoprotective/immunoregenerative therapy

Zorzopulos J, Opal SM, Hernando-Insúa A, Rodriguez JM, Elías F, Fló J, López RA, Chasseing NA, Lux-Lantos VA, Coronel MF, Franco R, Montaner AD, Horn DL

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Immunomodulatory oligonucleotide IMT504: Effects on mesenchymal stem cells as a first-in-class immunoprotective/immunoregenerative therapy

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Abstract

The immune responses of humans and animals to insults (*i.e.*, infections, traumas, tumoral transformation and radiation) are based on an intricate network of cells and chemical messengers. Abnormally high inflammation immediately after insult or abnormally prolonged pro-inflammatory stimuli bringing about chronic inflammation can lead to life-threatening or severely debilitating diseases. Mesenchymal stem cell (MSC) transplant has proved to be an effective therapy in preclinical

studies which evaluated a vast diversity of inflammatory conditions. MSCs lead to resolution of inflammation, preparation for regeneration and actual regeneration, and then ultimate return to normal baseline or homeostasis. However, in clinical trials of transplanted MSCs, the expectations of great medical benefit have not yet been fulfilled. As a practical alternative to MSC transplant, a synthetic drug with the capacity to boost endogenous MSC expansion and/or activation may also be effective. Regarding this, IMT504, the prototype of a major class of immunomodulatory oligonucleotides, induces *in vivo* expansion of MSCs, resulting in a marked improvement in preclinical models of neuropathic pain, osteoporosis, diabetes and sepsis. IMT504 is easily manufactured and has an excellent preclinical safety record. In the small number of patients studied thus far, IMT504 has been well-tolerated, even at very high dosage. Further clinical investigation is necessary to demonstrate the utility of IMT504 for resolution of inflammation and regeneration in a broad array of human diseases that would likely benefit from an immunoprotective/immunoregenerative therapy.

Key words: Immunohomeostasis; Immunoprotection; Immunoregeneration; Inflammation; Mesenchymal stem cells; IMT504

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Core tip: Mesenchymal stem cell (MSC) transplant has been demonstrated to be an effective therapy in pre-clinical studies evaluating a vast diversity of inflammatory conditions. However, in clinical trials of transplanted MSCs, the expectations of great medical benefit have not yet been fulfilled. In this regard, IMT504, the prototype of a major class of immunomodulatory oligonucleotides, induces *in vivo* expansion of MSCs, resulting in a marked improvement in preclinical models of neuropathic pain, osteoporosis, diabetes and sepsis. IMT504 is easily manufactured and has an excellent preclinical safety record. Further clinical investigation is necessary to demonstrate the utility of IMT504 for resolution of inflammation and regeneration in a broad array of human diseases that are likely to benefit from an immunoprotective/immunoregenerative therapy.

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INTRODUCTION

Homeostasis (from the Greek: Homeo, meaning

unchanging + stasis, meaning standing), is a concept that goes back to the old Greek philosophers who believed that harmony was a fundamental attribute of life and health. Empedocles (495-435 BC) hypothesized that all material comprised elements that were in active opposition or association, and that equilibrium was a necessary condition for subsistence of living entities. Thereafter, Hippocrates (460-375 BC) stated that healthiness is the tuneful equilibrium of the components of the body, and disease is the disorganized relationship of these components^[1,2]. Lately, Claude Bernard (1813-1878) specified that "All of the vital mechanisms, however varied they may be, always have one goal: To maintain the uniformity of the conditions of life in the internal environment (milieu intérieur)"^[3]. Finally, Cannon^[4] (1871-1945) expanded Claude Bernard's idea of constancy of the "milieu intérieur", naming his theory "homeostasis".

According to Cannon, homeostasis was a number of coordinated changes in the internal environment, leading to the preservation of physiological parameters within defined limits. These parameters encompassed temperature, pH, blood pressure and many others. Furthermore, in Cannon's view, homeostasis constancy requires communication among intelligent sensors able to identify unacceptable deviations. This concept of homeostasis is the most widely accepted nowadays, owing to its simplicity and physiologic rationale.

The immune system contributes to homeostasis by protecting the organism from an invasion by foreign organisms, such as bacteria, fungus, virus and parasites, and by participating in the defense of the organism against tissue damage caused by trauma, cancer or metabolic disorders such as diabetes. The immune response is biphasic, with the first phase represented by the inflammatory reaction, which aims for the prompt elimination of the causes of body aggression. Inflammatory signals include cytokines, chemokines, biogenic amines and eicosanoids that induce changes in diverse processes ranging from alterations in local vascular responses to abnormal rise in body temperature. Thus, acute inflammatory signals are antagonists of the normal homeostatic signals^[5]. The second phase of the immune response aims to restore the normal homeostatic parameters. This phase includes the clearing of debris from the "battlefield" created by invading pathogens and phagocytic cells, and then the reconstitution of tissue integrity and normal function.

In order to proceed from the initial inflammatory phase to the reconstitution phase, a switch command needs to be turned on. Failure to make this switch results in chronic inflammation and consequently in diseases such as autoimmunity (*i.e.*, diabetes, multiple sclerosis, lupus erythematosus) and neurodegenerative diseases (*i.e.*, Alzheimer's disease). However, termination of acute inflammation too early presents the risk of inadequate clearance of pathogenic microorganisms that can result in chronic infection. Therefore, gaining an understanding of the nature of the switching mechanism that connects

the first and second phase of the immune response is important for the finding of new efficient treatments.

Over the last few years, numerous studies have identified mesenchymal stem cells (MSCs) as the essential elements in this switching mechanism^[6], since transplant of autologous MSCs expanded *in vitro* or even allogenic MSCs results in significant salutary effects in animal models representing various inflammatory diseases^[7-9]. On the other hand, in 2007, we discovered that treatment of rats with a novel class of immunomodulatory oligonucleotides (ODNs) (PyNTTTGT ODNs) lacking CpG motifs, induces MSC expansion in bone marrow and blood, thus markedly increasing the therapeutic potential of the autologous MSC pool during pathologic conditions^[10]. This discovery greatly advances the development of defined, easy-to-produce and fully-controllable pharmaceuticals for treatment of inflammatory diseases. Such an exciting prospect as the one suggested by these studies prompted us to review the relevant information in the field of immunoprotection and immunoregeneration mediated by MSCs or ODNs of the PyNTTTGT class.

MSCS AND IMMUNOMODULATION

MSCs are non-embryonic multipotent cells characterized by the capability to differentiate into mesodermal cell, for instance osteoblasts, chondroblasts and adipocytes^[11,12]. MSCs are resident of bone marrow, adipose tissue, umbilical cord blood and may other tissues^[13-15]. These cells do not express class I or class II major histocompatibility complexes, thereby permitting adoptive transfer of MSCs between hosts without inducing acute rejection.

In addition to their progenitor cell properties, phenotypical plastic MSCs are able to interrelate with constituents of the immune system, exhibiting anti-inflammatory or pro-inflammatory properties depending on the milieu composition^[16-18]. In general, MSCs adopt a pro-inflammatory phenotype (MSC₁) during early microbial invasion or trauma, when the concentration of pro-inflammatory cytokines in the milieu is relatively low. Some important effects of MSC₁ at the damaged body site are stabilization of a pro-inflammatory classic phenotype (M₁) in resident macrophages and activation of antimicrobial properties of neutrophils^[19-23].

As inflammation proceeds, pro-inflammatory cytokines accumulate up to a critical level that switches differentiation of MSCs to an anti-inflammatory phenotype (MSC₂). Abundant information has been published on the relationship between MSC₂ and resolution of the inflammatory setting, and tissue protection and repair^[24-34]. Some of the well-known anti-inflammatory effects mediated by MSC₂ are skewing macrophages to the M2 immunosuppressive alternative phenotype^[35-41], promoting T cells to T regulatory (T_{reg}) cell differentiation^[42-48], skewing monocyte-derived dendritic cells to a regulatory phenotype^[49-54], inhibiting neutrophil influx and respiratory burst while maintaining

or even increasing its phagocytic capacity^[55-59], inhibiting mast cell degranulation^[60-62], and inhibiting pro-inflammatory activities of T cells^[63-72], natural killer (NK) cells^[73-79] and B cells^[80-84]. Furthermore, throughout the numerous reports describing the regulatory role of MSCs attenuating (at some point) inflammation, several intercellular molecular signals have consistently emerged as relevant. For example, the cytokines interferon-gamma (IFN- γ), interleukin (IL-6) and tumor necrosis factor-alpha (TNF- α), and also stimulation of the toll-like receptor (TLR)3 and TLR4 have been proposed as main signals for switching MSC differentiation to its anti-inflammatory and pro-resolving differentiation stage^[85].

Once differentiated into the anti-inflammatory and pro-resolving phenotype MSC₂, MSC communication with other cells is mediated by molecular signals such as prostaglandin E₂ (PGE₂), indoleamine 2,3-dioxygenase (IDO), TNF-inducible gene 6 protein (TSG-6), hepatic growth factor (HGF) and transforming growth factor-beta 1 (TGF- β 1). PGE₂ is a bioactive lipid with early and late effects in the setting of inflammation. In the early stages, PGE₂ stimulates vasodilatation, relocation and activation of macrophages, mast cells and neutrophils. Later on, PGE₂ promotes differentiation of macrophages and monocytoïd dendritic cells to an anti-inflammatory phenotype that suppresses NK cell and neutrophil inflammatory function and mast cell degranulation^[86]. Variances in sensitivity, desensitization and activation of different signaling pathways among several PGE₂ receptors accounts for this adaptable pattern of responses at different stages of the immune response^[87]. TGF- β 1 also presents biphasic activities, since its strong chemoattractive properties brings about a rapid incoming of T cells, granulocytes and macrophages that can contribute to inflammation but can also exert a potent anti-inflammatory response by constraining the synthesis of inflammatory cytokines and stimulating differentiation of naïve T cells to T_{reg} cells^[88]. IDO is an intracellular enzyme that catabolizes the production of kynurenine from tryptophan. Induction of IDO in the inflammatory setting results in arrest and functional anergy of CD8+ T cells, inhibition of differentiation of T helper (Th) cells to Th₁₇ cells and activation of differentiation to T_{reg} cells^[89]. TSG-6 is an anti-inflammatory protein secreted by MSCs in response to inflammatory cytokines (*i.e.*, IL-1 and TNF- α) that mediates suppression of dendritic cell maturation and function^[90]. HGF is a morphogenic and growth factor secreted by MSCs that also has anti-inflammatory activity by inhibition of the production of pro-inflammatory cytokines and by stimulation of macrophage differentiation to the M2 phenotype^[91].

Figure 1 displays a highly simplified representation of interactions between MSCs and other cells of the immune system during the anti-inflammatory phase of the immune defensive response. In addition, anti-inflammatory MSCs directly or indirectly interact with resident cells at the site of inflammation, for example with oligodendrocytes in the central nervous system,

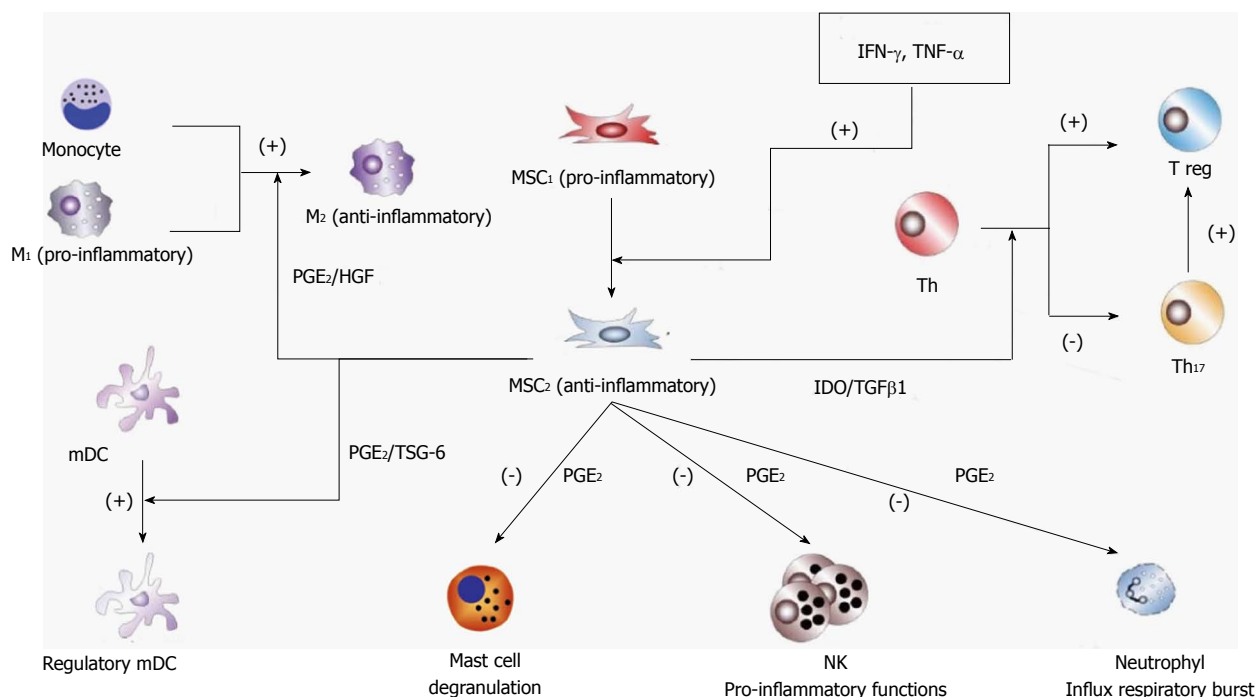


Figure 1 Mesenchymal stem cell immunosuppressive regulatory effects. MSCs are polarized to an immunosuppressive stage (MSC2) by a high relative concentration of pro-inflammatory cytokines such as IFN- γ and TNF- α . MSC2 induce macrophage polarization of monocytes and pro-inflammatory macrophages (M1) to the immunosuppressive stage M2 by secreting immunomodulatory mediators such as PGE2 and HGF. MSC2 also induce differentiation of Th and Th17 to T regulatory cells (Treg) by secretion of TGF- β 1 and indoleamine 2,3-dioxygenase (IDO). Furthermore, MSC2 induce differentiation of mDCs to a regulatory anti-inflammatory stage (mDCreg), inhibit mast cell degranulation, inhibit NK cell pro-inflammatory functions and suppresses neutrophil respiratory burst. MSC2-derived PGE2 contributes to all of these effects. Other cytokines that have been implicated in at least some of the MSC2 immune-suppressive effects are IL-6 and GM-CSF^[6]. MSC: Mesenchymal stem cell; IFN- γ : Interferon-gamma; TNF- α : Tumor necrosis factor-alpha; PGE2: Prostaglandin E2; Th: T helper; HGF: Hepatic growth factor; TGF- β 1: Transforming growth factor-beta 1; mDCs: Monocyte-derived dendritic cells; NK: Natural killer; IL-6: Interleukin-6; GM-CSF: Granulocyte macrophage-colony stimulating factor.

with osteoblasts and osteoclasts in bones, with beta cells in pancreas, *etc.* Therefore, the ability to respond under such diverse circumstances requires a highly adaptable cell, such as MSCs, in order to orchestrate the appropriate response.

PROSPECTIVE THERAPEUTIC USE OF MSC TRANSPLANT

The central role of MSCs in maintaining tissue homeostasis serves as the basis for their therapeutic application in many diverse inflammatory disorders. A large number of reported studies representing a wide spectrum of diseases reinforce this expectation. For example, MSC transplant has proven to be beneficial in preclinical as well as clinical studies of heart disease^[92-105], renal disease^[106-142], lung disease^[143-159], liver disease^[160-175], neural system disease^[176-204], bone damage^[205-227], skin wound healing^[228-244], autoimmune disease^[245-265], infectious diseases and sepsis^[266-287], allergies and asthma^[288-306], graft vs host disease^[307-325] and diabetes^[326].

Despite the current enthusiasm about the broad potential clinical use of MSC transplant, some concerns have been growing about some potential issues as follows.

Economic

MSC-based treatments might be expensive if founded on autologous cells because of the need to take a biopsy for each patient, grow the cells *in vitro*, and perform the quality testing previously to the use of MSC for treatment. Furthermore, it is not sure that this process would produce enough cells as needed or if these cells would retain their phenotypical and functional characteristics after subculture. Convenient substitutes of autologous MSCs are allogeneic MSCs because they do not present immunologically significant surface molecules and in consequence do not provoke significant immune rejection to cell transplantation. Therefore, allogeneic MSC can be multiplied, aliquoted and stored beforehand and used when needed for treatment. Still, several regulatory and safety issues concerning allogeneic MSCs should be resolved as discussed below.

Reproducibility

Consistent results with allogeneic MSC therapies are possible if the different cell batches are constant within certain prefixed limits. Nevertheless, each allogeneic MSC batch is originated from a different donor. This fact results in substantial variation among the cell batches excluding establishment of a master cell bank. Furthermore, the starting material (*i.e.*, bone marrow

aspirate) consists in several cell types, and current techniques to isolate MSCs can rarely result in a pure cell preparation. This can be solved deriving the batch from a single cell; a fact that implies a long growth process that could result in undesirable random mutations.

Safety issues

MSC prepared from human tissues might hold retroelements, retroviruses and other viruses, and many other pathogens. A handful of these pathogens can be detected using current assays. Microbial contaminants may also upset therapeutic potency of MSCs. In addition, the use of fetal calf serum during cell growing culture raises concern regarding transmission of prion-associated diseases.

Rationality of MSC treatment to stimulate tissue repair rest on the hypothesis that endogenous repair prompted by MSC expansion, activation and relocation from the patient's own MSCs reservoirs is deficient in numerous pathological conditions. A reasonable alternative to cell infusion could be the use of a synthetic medicine aimed to stimulate expansion, activation and relocation of the patient endogenous stem cells, as long as the disease does not permanently altered these endogenous cells. Development of a medicine like this may solve most, of the above-stated difficulties connected with therapeutic applications of MSC transplant.

In this regard, our research group has pursued study for several years on the properties of a major class of immunostimulatory ODNs with the capacity to stimulate *in vitro* and *in vivo* MSC expansion. Preclinical studies indicate that these synthetic drugs are safe and competent in the treatment of several of the disorders that are responsive to MSC transplant. General properties of the prototype of these ODNs, named IMT504, will be briefly described in the following sections, with special emphasis on the ability of IMT504 to promulgate endogenous recruitment of MSCs for regenerative medicine.

IMMUNOMODULATORY OLIGONUCLEOTIDE IMT504 AND INFLAMMATORY DISEASE

Oligonucleotides with regulatory activities on the immune system may be categorized into two major classes: (1) CpG ODNs, that include at least one CpG dinucleotide^[327]; and (2) PyNTTTTGT ODNs, that include at least one PyNTTTTGT octanucleotide in (Py: Pyrimidine; N: Adenine, Cytosine, Thymidine or Guanine; T: Thymidine; G: Guanine)^[328]. ODNs of both classes have as target cells B-cells and/or plasmacytoid dendritic cells (PDCs).

The seal of CpG ODNs is their capability to stimulate secretion of IFN- α by PDCs interacting with the TLR9^[329,330], a characteristic that is absent in members of

the PyNTTTTGT class. On the other hand, hallmarks of the PyNTTTTGT class are induction of an efficient release into the milieu of granulocyte macrophage colony-stimulating factor (GM-CSF) by NK and natural killer T (NKT) cells in collaboration with IL-2^[331] and stimulation of MSCs^[10], characteristics that are absent or poorly expressed in CpG ODNs. Interestingly, IFN- α inhibited the GM-CSF secretion stimulated by PyNTTTTGT ODNs, and reciprocally these ODNs inhibit the excretion of IFN- α stimulated by CpG ODNs *via* TLR9 in PDCs^[331]. Therefore, this mutual interference between ODNs of the major classes of immunostimulatory ODNs suggested that they stimulate different and incompatible immune response pathways^[331].

Participation of MSCs in the pathway stimulated by PyNTTTTGT ODNs prompted us to hypothesize that these ODNs may modulate the inflammatory process, thereby stimulating the switch from the pro-inflammatory to the anti-inflammatory reconstructive stage of the immune response. To test this hypothesis, IMT504, the prototype of the PyNTTTTGT ODN class, was assayed as a therapeutic agent in animal models representing diverse medical conditions in which an MSC transplant had proven to be useful. The chosen animal models were of neuropathic pain, osteoporosis, diabetes and sepsis. A brief description of these preclinical studies is provided below.

Neuropathic pain

Neuropathic pain is a chronic, excruciating pain triggered by a injury or disease of the somatosensory system^[332]. Typical symptoms of neuropathic pain include allodynia (an answer to painful stimulation that does not usually provoke discomfort), hyperalgesia (augmented pain induced by stimuli that usually provoke pain), and spontaneous pain^[333]. While pain represents an adaptive response, acting as a protective mechanism that inform an organism of actual or potential tissue injury, neuropathic pain is thought as a maladaptive answer of the nervous system to harm^[334]. MSC transplantation has proven to be effective for the treatment of neuropathic pain in several preclinical studies^[335-342]. In addition, parenteral treatment with IMT504 has been shown to ameliorate neuropathic pain in a rat model of peripheral nerve lesion even when administered several days after nerve injury^[343] (Figure 2).

Osteoporosis

Osteoporosis is a medical condition characterized by decreased bone strength that results in frequent fractures. Mechanistically, osteoporosis results from a pathological increase of the activity rate of osteoclasts vs osteoblasts^[344]. Usually, osteoporosis has been considered an exclusive endocrine disease; however, it is now well established that continuing inflammation plays an important role in the osteoporosis development^[344,345]. Pro-inflammatory cytokines (*e.g.*, IL-6 and TNF- α), stimulate osteoclastogenesis and inhibit

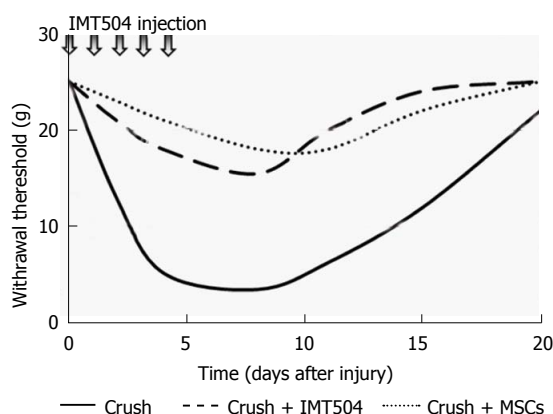


Figure 2 Effect of IMT504 or mesenchymal stem cell treatment on the development of mechanical allodynia in rats. Sciatic nerve crush induced a significant decrease in paw withdrawal threshold to the von Frey filaments. It is noticeable that the administration of either IMT504 or MSCs prevents the development of allodynia. Experimental details are described in Coronel *et al.*^[343]. MSC: Mesenchymal stem cell.

osteoblastogenesis and anti-inflammatory cytokines (e.g., IL-4 and IL-10), inhibit osteoclastogenesis^[344]. There are currently only a few preclinical studies that have been published on the effect of MSC transplant in osteoporotic animals, and results in these reports are encouraging^[346,347]. Furthermore, in a study performed in an ovariectomized rat model of osteoporosis, we observed that parenteral treatment with IMT504 results in a remarkable recovery of the bone structure, as indicated by morphometric characteristics such as trabecular volume, trabecular density, trabecular thickness and trabecular distance in the femur head (Figure 3).

Diabetes

Diabetes is a group of metabolic illnesses characterized by high blood glucose levels and altered metabolism of sugars, fatty acids and proteins because of faults in insulin secretion, activity, or both^[348]. Type 1 diabetes results from deficient insulin production by the pancreas and its cause is unknown. Symptoms are polyuria, polydipsia, continuous hunger, weight loss, visual alterations and fatigue. Type 1 diabetes patients are susceptible to a potentially lethal state of diabetic ketoacidosis.

Type 2 diabetes begins with the fail of cells to properly react to insulin. Symptoms are similar but usually less marked than those of type 1 diabetes. Type 2 diabetes patients rarely results in ketoacidosis^[349].

Although the cause of type 1 diabetes is unknown, contribution of the immune system in pancreas and other organs damage in type 2 diabetes is unquestionable^[350]. The key pathogenic event appears to be damage of pancreatic β cells caused by the attack of autoreactive cytotoxic T cells resulting in chronic inflammation of the pancreatic islets^[351].

In type 2 diabetes, a state of chronic inflammation encompassing innate and adaptive immune responses,

is in general accepted to be the primary alteration^[352]. Since islet inflammation contributes to the loss of functional β cells in both type 1 and type 2 diabetes, anti-inflammatory therapies have emerged as a reasonable option to current treatments. In particular, MSC transplant as a therapy in animal models of type 1 and type 2 diabetes resulted effective^[353-356]. In these studies, improvement of the glucose metabolism and regeneration of pancreatic islets were observed. Furthermore, parenteral treatment with IMT504 also markedly reversed pancreatic damage in a rat model of diabetes induced by one high-dose administration of streptozotocin^[357]. A striking recovery of islet number and structure accompanied by lowering of glucose and rising of insulin concentration reaching normal levels was observed in diabetic animals during and after the treatment (Figure 4). Study of histological markers for pancreatic progenitor cell proliferation and differentiation and for active angiogenesis indicated that stimulation of the remaining resident pancreatic islet cells might be critical for success of the IMT504 treatment.

Sepsis

Sepsis is a syndrome of dysregulated systemic immune responses to an infection or to microbial pathogenic components^[358]. Diabetes mellitus, lymphoproliferative disease, hepatic cirrhosis, extensive burning, severe trauma, use of intravenous or vesicular catheters, prosthesis and treatments with immunosuppressive medicines or intravenous drugs are frequent causes that contribute to acquisition of infections resulting in sepsis.

Stimuli prompting sepsis can be exogenous (*i.e.*, infectious) or endogenous (*i.e.*, severe trauma) resulting in gut hypoperfusion, impaired epithelial barrier function and translocation of luminal bacteria and/or their toxins into the systemic circulation. Pathogen-associated molecular patterns and damage-associated molecular patterns are recognized by pattern recognition receptors. These alarm signals activate systems in charge of keeping homeostasis. However, during sepsis, this system becomes dysregulated, leading to multiple organ damage.

During a first phase of sepsis, oxygen and nitrogen reactive forms accumulate. Some symptoms corresponding to this period include tachycardia, fever and neutrophilia. This is quickly followed by a marked elevation of proinflammatory cytokines and chemokines in plasma as well as the migration of polymorphonuclear leukocytes, monocytes and lymphocytes to affected tissues. Owing to this dramatic presentation, the prevalent and long-time definition for sepsis has been that of an uncontrolled inflammatory response. However, a number of recent observations have led to a redefinition of sepsis^[359], bringing about the idea that in sepsis there exist successive pro-inflammatory and anti-inflammatory (immunosuppressive) periods. Even though some patients die during the first pro-inflam-

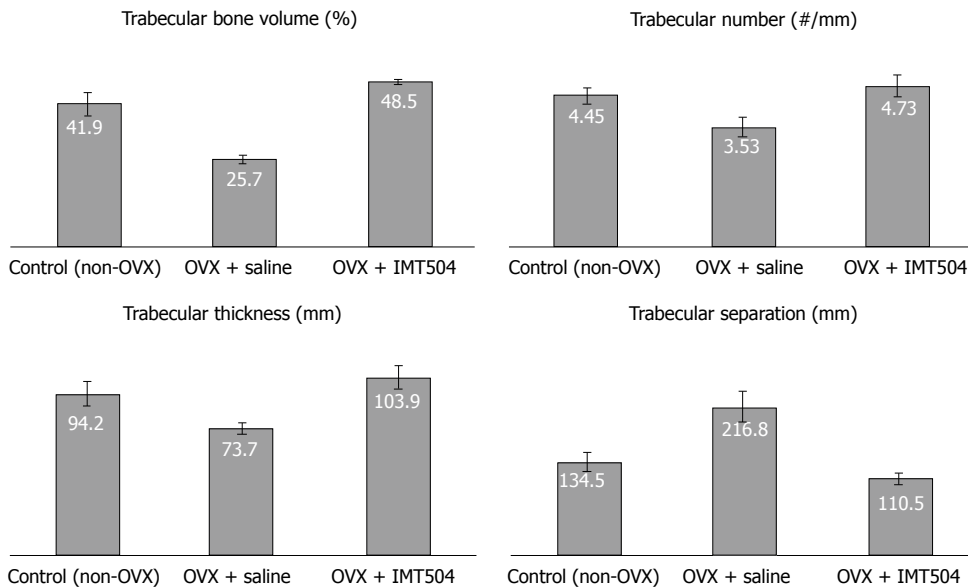


Figure 3 Effect of the IMT504 treatment on bone structure in ovariectomized (osteoporotic) rats. Female Sprague-Dawley 16-wk-old rats underwent ovariectomy (OVX). When animals were 1-year-old, half of them (treated group) received a subcutaneous dose of IMT504 (20 mg/kg per dose in saline) injected daily for 5 successive days. The other half received saline under the same scheme (non-treated group). The treatment was repeated 30 d later. A group of non-OVX rats served as control. Body weight and general health was measured weekly. One month after the last treatment, animals were euthanized and femurs dissected, decalcified and embedded in paraffin. Slides of 0.5- μ m sections of the distal femur were generated using a Leica RM2145 microtome, stained with hematoxylin and eosin, and examined by light microscopy. Digital images were recorded with a Nikon Coolpix 4500 camera at 16.5-fold magnification under a Leica MZ16A stereomicroscope. Three fields in each slide were evaluated, totaling a combined area of 3 mm². Trabecular bone was identified, and its perimeter and area measured. Histomorphometric analysis was performed using the Image Pro-Plus 4.5 software and standard histomorphometric parameters calculated (our unpublished results).

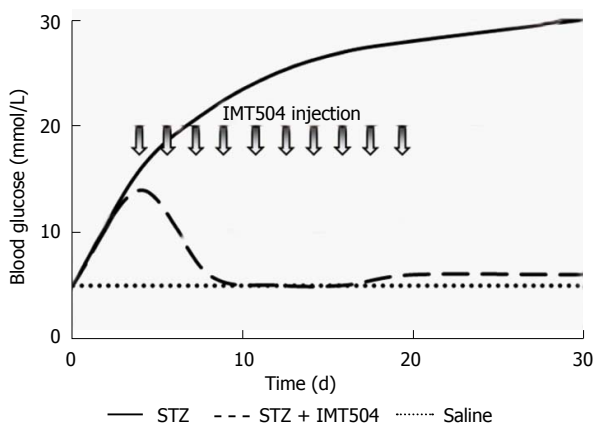


Figure 4 IMT504 treatment induced a marked recovery of the diabetic condition in streptozotocin-treated rats. Arrows indicate IMT504 treatment, which consisted of daily subcutaneous injections containing 4 mg of IMT504 over 10 successive days. Experimental details are described in Bianchi *et al.*^[357]. STZ: Streptozotocin.

matory period, due to septic shock, most patients survive it presently^[360]. The great majority of deaths occur during the immunosuppressive period, which in general starts between the second and third day of sepsis and could persist for several weeks. In spite of antibiotic treatment and strong medical supportive care, many patients cannot eradicate the infection and may acquire secondary intra-hospital infections^[361].

MSC transplant has been protective in preclinical animal models of polymicrobial sepsis^[282,270] as well as

in infections caused by bacterial strains of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*^[266,284,362]. The protective role of MSCs in sepsis has been mainly attributed to their broad paracrine modulatory properties^[269]. On the other hand, remarkable protection against *Pseudomonas* infection was obtained in neutropenic rats in response to IMT504 treatment^[363]. Protection was 90%-100% using either early or late intervention after infection on par with antibiotic treatment (Figure 5). IMT504 treatment resulted in a marked decrement in serum IL-6 and in bacterial load in organs such as lungs, liver and spleen.

PRACTICAL CONSIDERATIONS REGARDING THE PROSPECTIVE CLINICAL USE OF IMT504 FOR TREATMENT OF INFLAMMATORY DISEASES

IMT504 is a drug with a well-defined formula that is relatively easy to synthesize using a rapid automatic process under GMP conditions and at reasonable cost if large quantities are required. In addition, formulations of IMT504 are not problematic because IMT504 is highly soluble. Additionally, once injected using different routes, IMT504 has a rapid and broad distribution^[364]. Moreover, because IMT504 has good thermal stability, extreme conditions of transport and storage are not

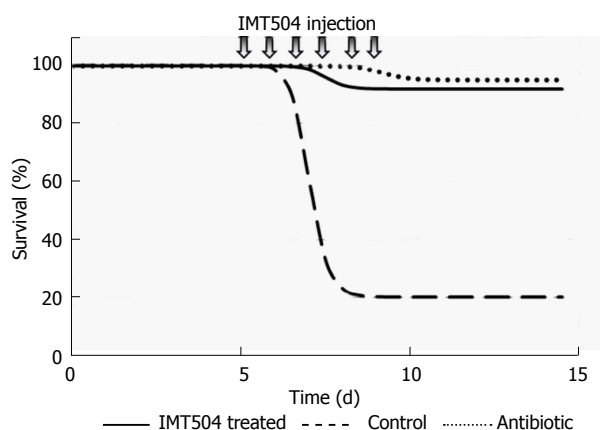


Figure 5 IMT504 protects neutropenic animals from fatal *Pseudomonas aeruginosa* bacteremia and sepsis. Kaplan-Meier survival plot representing IMT504 monotherapy vs antibiotic (cefepime) monotherapy vs control. IMT504 daily doses were started at day 5 after bacterial infection. Arrows indicate IMT504 treatment, which consisted of subcutaneous injections containing 50 g of IMT504 over 5 successive days. Experimental details described in Chahin *et al.*^[363].

necessary. Finally, IMT504 preclinical toxicity studies performed in several animal species, including non-human primates, indicate that IMT504 is a very safe drug with few secondary effects that are well-tolerated and within the therapeutic range of this agent^[364,365].

WHAT DO WE KNOW ABOUT THE MECHANISM OF ACTION OF IMT504 ON THE IMMUNE SYSTEM?

Known direct cell targets of IMT504 are B cells, PDCs, CD56⁺ cells (NK and NKT cells) and MSCs^[10,328,331].

B cells

B cells contribute to the immune response by producing antibodies and stimulating T cell activation^[366]. Besides, B cells can act as professional antigen-presenting cells (APCs) and B cell antigen presentation is essential for specific CD4⁺ T cell expansion, memory development and cytokine secretion^[367,368]. CD80, CD86, and CD40 surface components of B cells are essential for optimal T cell activation^[369]. Furthermore, in inflammation and autoimmunity, B cells exert an immunomodulatory role in part by IL-10 production and secretion^[370]. *In vitro* stimulation of human immature B cells with IMT504 results in cell proliferation, MHC I, MHC II, CD40, CD80 and CD86 cell surface expression, immunoglobulin secretion, and IL-6 and IL-10 secretion^[328]. Furthermore, upon stimulation with IMT504, B cell transcripts for most of the components of the proteasome are significantly augmented (our unpublished results). Most of these effects indicate that IMT504 incubation empowers B cells for competent presentation of antigens to CD4⁺ T cells. In line with this, addition of IMT504 to different vaccines greatly increases their activity^[371-373]. However, the strong secretion of IL-6 and IL-10 induced by

IMT504 suggests that IMT504-activated B cells may also participate in regulation of the immune response.

PDCs

PDCs are dendritic cells specialized in producing type I IFNs when stimulated by nucleic acids through TLRs 7 and 9^[374]. Additionally, PDC stimulation by nucleic acids results in surface expression of MHC I, MHC II, CD40, CD80 and CD86^[375]. Consequently, PDCs can present antigens to CD4⁺ T cells, leading to activation or tolerance depending on the context^[375,376]. PDCs are also involved, by unrestrained IFN type I secretion, in several inflammatory autoimmune diseases such as multiple sclerosis, psoriasis, systemic lupus erythematosus and inflammatory bowel disease^[377]. *In vitro*, stimulation of human immature PDCs with IMT504 also results in surface expression of MHC I, MHC II, CD40, CD80 and CD86^[328]. However, in contrast with CpG ODNs, IMT504 does not induce IFN type I secretion. Furthermore, incubation with IMT504 inhibits PDC IFN type I secretion induced by CpG ODNs^[331]. Interestingly, this inhibition of the IFN type I secretion allows activation of CD56⁺ (NK and NKT) cells by IMT504 in collaboration with IL-2, resulting in strong secretion of IFN- γ , TNF- α and GM-CSF^[331].

CD56⁺ (NK and NKT) cells

NK cells are innate lymphoid cells involved directly in the immune protection through cytotoxicity and cytokine secretion, and indirectly by modulating APCs and T cells^[378]. The cytotoxic activity of NK cells depends on the release of lytic molecules toward target cells. NK cells can stimulate inflammation by excreting cytokines (e.g., IFN- γ and TNF- α); however, they can also limit inflammation and autoimmunity^[379,380].

On the other hand, NKT cells specialized in recognition of lipid antigens presented by an MHC I-like antigen (CD1d). NKT cells also are able to modulate the immune responses involved in inflammation and autoimmunity^[381]. Incubation of human PBMCs with IMT504 results in strong secretion of IFN- γ , TNF- α and GM-CSF, providing that IL-2 is present in the milieu^[331]. IL-2 induces synthesis of the cytokines, and the presence of an ODN is necessary for their efficient secretion. CD56⁺ (NK and NKT) cells are responsible for the cytokine secretion and IFN- α inhibits the process. Induced cytokine secretion depends on two different IMT504 activities: (1) inhibition of the TLR9 dependent IFN- α secretion from PDCs; and (2) activation of a pathway of cytokine secretion presumably similar to the one described by Rao *et al.*^[382]. This last effect does not depend on the nucleotide sequence since ODNs with very diverse compositions were able to stimulate cytokine secretion when acting on purified CD56⁺ cells^[331].

Figure 6 shows a schematic representation of the likely IMT504 effects leading to defensive immune activation as well as resolution of excessive inflammation by MSC expansion and secretion of cytokines necessary

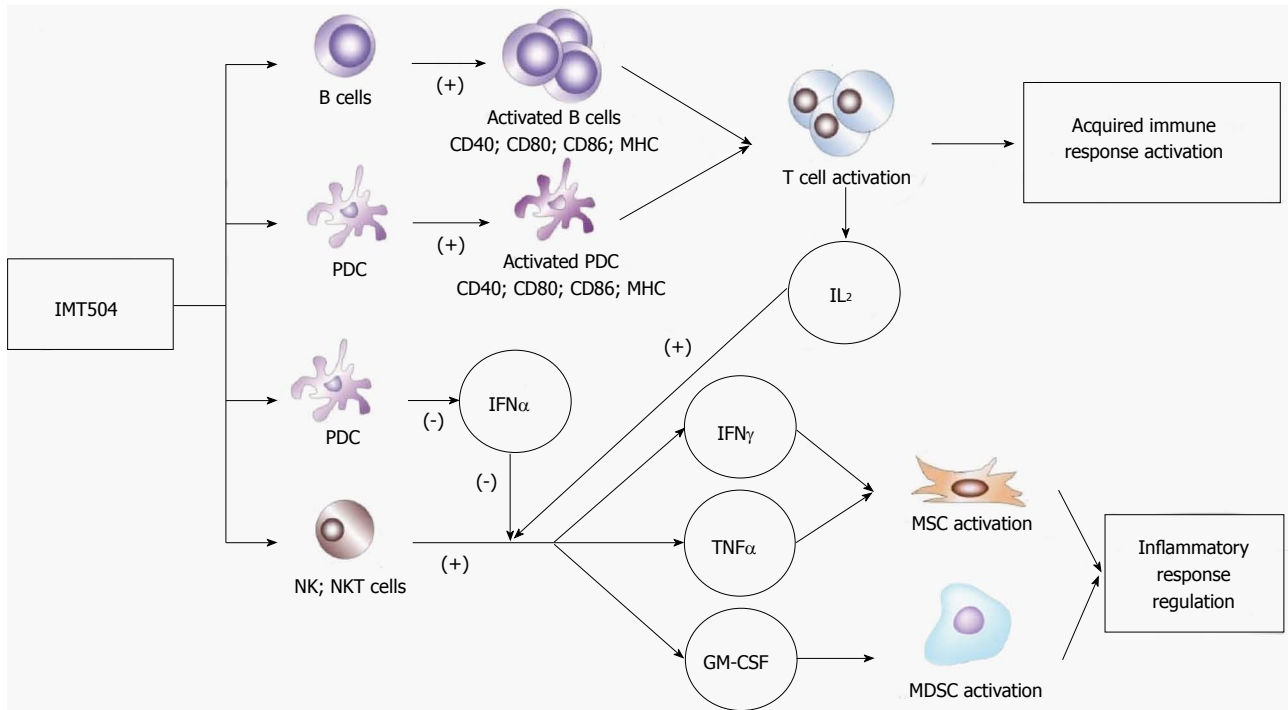


Figure 6 IMT504 effects on the immune system. Primary targets of IMT504 are B lymphocytes (B), PDCs, NK and NKT cells. IMT504 acting on B and PDCs induces a phenotype of antigen presenting cells, which in the presence of an appropriate antigenic stimulus initiates a strong adaptive immune response^[371-373]. On the other hand, IMT504 acting on NK and NKT cells, in collaboration with IL-2, induces the strong secretion of IFN- γ and TNF- α that can induce MSC immunosuppressive properties and of GM-CSF that can activate MDSCs. This immunosuppressive pathway is inhibited by the presence of IFN- α . Reciprocally, IMT504 inhibits IFN- α secretion by PDCs. Therefore, activation of this immunosuppressive pathway depends on the balance between IMT504 activity and activity of IFN- α inducers that are present^[331]. PDCs: Plasmacytoid dendritic cells; NK: Natural killer; NKT: Natural killer T; IL-2: Interleukin-2; IFN- γ : Interferon-gamma; TNF- α : Tumor necrosis factor-alpha; GM-CSF: Granulocyte macrophage colony-stimulating factor; MDSCs: Myeloid-derived suppressor cells.

for MSC differentiation to the MSC₂ anti-inflammatory stage. This scenario is congruent with the results of the above-described IMT504 preclinical assays involving animal models of neuropathic pain, osteoporosis, diabetes and sepsis.

CONCLUSION

The immune homeostatic response of animals to aggression (infections, traumas, tumoral transformation, radiation, *etc.*) is based on an intricate network of cells and chemical messengers. Abnormally high inflammation immediately after aggression or abnormally prolonged pro-inflammatory stimulus bringing about chronic inflammation are associated with life-threatening and severe debilitating diseases^[383]. In both cases, albeit with different urgency, therapeutic intervention to restore homeostasis of the immune system is necessary. Current interventions mainly rest on positive or negative action on a particular element of the immune network abnormally represented in a specific immune disorder. However, given the complexity of the immune network and the general pleiotropism of its components, the effect of such interventions is often poor or even contradictory with the "a priori" rationality^[384,385]. An exception is the transplantation of MSCs, which has demonstrated to be effective in preclinical studies representing a vast array of inflammatory conditions. Unfortunately, results

from clinical trials involving transplantation of MSCs, in general, have not fulfilled expectations. Cell dosing and/or cell preconditioning seem to be critical issues that should be further studied in order to improve human treatments. As an alternative to MSC transplantation, a synthetic drug with the capacity to boost human MSC expansion and/or activation *in vivo* may also be effective, while avoiding many of these problems.

Regarding this, we have reported that IMT504, the prototype of a major class of immunomodulatory ODNs, induces *in vivo* expansion and likely activation of MSCs. This effective endogenous recruitment of MSCs by IMT504 for regenerative medicine results in a marked improvement of animals' chronic suffering as well as acute inflammatory disorders such as neuropathic pain, osteoporosis, diabetes and sepsis. IMT504 can be easily synthesized, purified and mass produced, and has an excellent preclinical safety record. In the small number of patients studied thus far, IMT504 has been well-tolerated, even at very high dosage. Further clinical investigation is necessary to demonstrate the utility of IMT504 for resolution of inflammation and regeneration in a broad array of human diseases that are likely to benefit from immunoprotective/immunoregenerative therapy.

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Fifteen years of bone marrow mononuclear cell therapy in acute myocardial infarction

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Abstract

In spite of modern treatment, acute myocardial infarction (AMI) still carries significant morbidity and mortality worldwide. Even though standard of care therapy im-

proves symptoms and also long-term prognosis of patients with AMI, it does not solve the critical issue, specifically the permanent damage of cardiomyocytes. As a result, a complex process occurs, namely cardiac remodeling, which leads to alterations in cardiac size, shape and function. This is what has driven the quest for unconventional therapeutic strategies aiming to regenerate the injured cardiac and vascular tissue. One of the latest breakthroughs in this regard is stem cell (SC) therapy. Based on favorable data obtained in experimental studies, therapeutic effectiveness of this innovative therapy has been investigated in clinical settings. Of various cell types used in the clinic, autologous bone marrow derived SCs were the first used to treat an AMI patient, 15 years ago. Since then, we have witnessed an increasing body of data as regards this cutting-edge therapy. Although feasibility and safety of SC transplant have been clearly proved, it's efficacy is still under dispute. Conducted studies and meta-analysis reported conflicting results, but there is hope for conclusive answer to be provided by the largest ongoing trial designed to demonstrate whether this treatment saves lives. In the meantime, strategies to enhance the SCs regenerative potential have been applied and/or suggested, position papers and recommendations have been published. But what have we learned so far and how can we properly use the knowledge gained? This review will analytically discuss each of the above topics, summarizing the current state of knowledge in the field.

Key words: Bone marrow stem cells; Acute myocardial infarction; Cell therapy; Cardiac regeneration; Remodeling

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Core tip: Since the first successful bone marrow stem cells transplantation performed 15 years ago in a patient with acute myocardial infarction, we have witnessed a mounting body of data as regards this cutting-edge therapy. During the reporting period, conflicting results have been stated, scientific papers have been under investigation, strategies

to enhance the stem cells regenerative potential have been applied and/or suggested, position papers and recommendations have been published. This review will analytically discuss each of the above topics, summarizing the current state of knowledge in the field.

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INTRODUCTION

The optimal management of acute myocardial infarction (AMI) still remains elusive, although it represents an illness with one of the highest morbi-mortality and one of the highest healthcare costs worldwide. The quick and efficient restoring of myocardial blood flow is the most appropriate strategy for reducing the size of the infarcted area. Even though standard-of-care therapy diminishes the area at risk to become necrotic, cardiac remodeling may occur in up to 60% of patients having suffered an AMI^[1-3]. The conventional available treatments (whether pharmacological, interventional or surgical)^[4] do not address the crucial issue of cell loss, thus being unable to completely prevent or reverse this pathological process which eventually leads to changes in size, shape, structure and function of the entire heart. One of the latest breakthroughs in this regard is stem cell (SC) therapy. By providing a potential source of new cells, heart function may be enhanced. Ideally, this process allows the replacement of non-functional cardiomyocytes and scar tissue with new fully functional contracting cells, as well as new blood vessels. Furthermore, transplanted SCs may secrete a variety of growth factors and cytokines, thereby enhancing myocyte survival and facilitating the migration of remote and/or resident cardiac SCs to the site of injury.

One of the first SC types which have been tested in clinical settings is autologous bone marrow SC. Since the first successful bone marrow SCs transplantation performed 15 years ago in a 46-year-old patient with AMI, we have witnessed a mounting body of data related to this effervescent domain: Conflicting results have been reported, scientific papers have been under investigation, strategies to enhance the SCs regenerative potential have been applied and/or suggested, position papers and recommendations have been published. A time line chart of accomplishments performed during the last fifteen years is depicted in Figure 1. But what have we learned so far and how can we properly use the knowledge gained? This review will analytically discuss each of the above topics, summarizing the current state of knowledge in the field.

HALLMARK CLINICAL TRIALS

Bone marrow is a very heterogeneous compartment with multiple SC populations with putative cardiac regenerative potential (e.g., hematopoietic SCs, mesenchymal SCs, endothelial progenitor cells, etc.).

The regenerative potential of adult autologous SCs after AMI was assessed for the first time in 2001 by a German group^[5]. They used unfractionated bone marrow mononuclear stem cells (BMMNCs), which contained both hematopoietic and nonhematopoietic cells, a protocol that was extensively used subsequently. After selective catheterization of the infarct-related artery, the BMMNCs suspension has been intracoronary injected. Ten weeks later, the infarct area had been notably reduced (from 24.6% to 15.7%); in addition, cardiac function had improved by 20%-30%. Accordingly, the authors concluded that intracoronary administration of human autologous adult BMMNCs is feasible in clinical settings and that it can promote myocardial regeneration after transmural infarction.

The following years were characterized by a series of small Phase I clinical trials whose primary achievement was demonstrating the feasibility and safety of this ground-breaking therapy^[6-10].

Since most of these studies have been comprehensively discussed in previous reviews^[11-14], we will briefly point out their main characteristics. What they have in common is the small number of patients enrolled (with or without a control group) and the assessment of left ventricular ejection fraction (LVEF) as a surrogate marker of cardiac function. Although not designed to evaluate the efficacy of the therapy, the early trials reported a beneficial effect on cardiac function as revealed by increased global or regional LVEF, reduced endsystolic LV volumes and enhanced perfusion within the infarcted area 4 to 6 mo after SC transplantation depending on study design.

The next logical step was the appearance of randomized clinical trials (RCT) designed to test whether this therapy works. A wide variety of RCT have been conducted in this regard, with number of patients ranging from 20^[15,16] to 204^[17], but not all studies successfully blinding the participants and/or caregivers^[15,16]. Studies varied also in terms of baseline LVEF, as well as diagnostic tests and procedures used to evaluate cardiac volumes and function. The most utilized imagistic method was cardiac echocardiography followed by cardiac magnetic resonance (CMR) - the "gold" standard for noninvasively characterizing cardiac function and viability, while LV angiography and single-photon emission computed tomography (SPECT) being exploited less frequently. Noteworthy, the timing of cell delivery after AMI, the quantity and quality of transplanted cells, as well as cell handling varied greatly, so is no wonder why apparently similar studies had different results.

Some of the hallmark studies using unfractionated bone marrow mononuclear SCs were conducted more

Fifteen years of bone marrow mononuclear cell therapy in acute myocardial infarction - time line chart

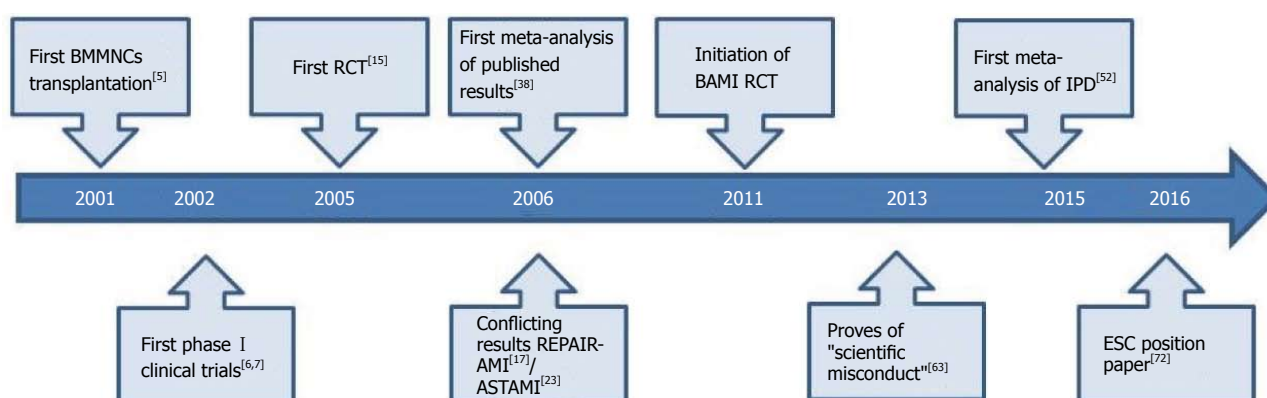


Figure 1 Fifteen years of bone marrow mononuclear cell therapy in acute myocardial infarction - time line chart. BMMNC: Bone marrow mononuclear stem cell; RCT: Randomized clinical trials; AMI: Acute myocardial infarction; IPD: Individual patient data.

than 10 years ago (Table 1). The BOOST study tested the usefulness of autologous BMMNCs intracoronary transfer 4.8 \pm 1.3 d after AMI^[10]. At baseline ($n = 60$), the two groups of patients were homogeneous in terms of LV volumes and function; 6 mo later, a mean global LVEF improvement of 6.7% in the cell therapy group and 0.7% in the control group (P value for between-group comparison = 0.0026) was documented, enhanced LV systolic function being predominantly witnessed in myocardial segments bordering the infarcted area.

Although significant augmentation of LV function after SCs transplant have been observed in the first months, this positive effect seems to be fading in time. Long-term benefit of SC therapy was assessed in BOOST surviving patients. Eighteen months after AMI ($n = 59$), there were no significant differences between groups as regards global LVEF ($P = 0.27$), although the speed to LVEF recovery was significantly higher in patients receiving SC transplant ($P = 0.001$)^[18].

Moreover, 5 years after randomization ($n = 56$), statistical analysis of data revealed no difference between groups with reference to cardiac dimensions or function. Repetitive CMR examinations indicated an evident dilatation of LV volumes, whereas LV function decreased during 61 mo follow-up^[19].

Reinfusion of enriched progenitor cells and infarct remodeling in acute myocardial infarction (REPAIR-AMI) - the largest study reported so far, also demonstrated the benefit of BMMNCs intracoronary infusion in patients with optimally treated AMI. From the 204 patients included, 103 were randomly assigned to placebo group and 101 to receive SC therapy. Both groups were well matched with respect to baseline characteristics, procedural characteristics of reperfusion therapy and associated pharmacological therapy during the study. Three to 7 d after successful stent implantation, cell suspension or placebo medium was injected in the infarct-related artery. Four months later, significant improvement in both global and regional LV function was documented in the cell treated group. Of note, the study led by Andreas Zeiher was the first trial to evaluate the interaction between

the BMMNCs treatment effect and the timing of cell delivery. Subgroup analysis revealed superior recovery of contractile function when cell infusion was administered on day 5 or later after PCI, while earlier administration - within 4 d after reperfusion therapy - had only minimal effects as regards LVEF improvement. Furthermore, intracoronary administration of BMC abolished LV end-systolic volume enlargement after the infarction.

Even though REPAIR-AMI was not powered to detect significant differences in major adverse clinical events between the cell therapy and control group, a reduction in the combined outcome of death, recurrence of MI, or any revascularization procedure was noticed^[17].

As opposed to BOOST trial - in which positive effects have faded in time, 2- and 5-year follow-up of REPAIR-AMI patients demonstrated a persistent reduction of the combined end point of death, recurrent MI and rehospitalization for heart failure in the BMMNCs group compared with placebo. In addition, 2 years after AMI, SC therapy was still associated with a significant improvement in regional left ventricular contractility of infarcted segments^[20,21].

The significant and longstanding positive effects of SC therapy were further confirmed by a study conducted by Bodo-Eckehard Strauer's group: The BALANCE Study (Clinical benefit and long-term outcome after intracoronary autologous bone marrow cell transplantation in patients with AMI) which randomized 124 patients to BMMNCs (62 patients) or control (62 patients) 7 \pm 2 d after AMI^[22]. The patients were followed-up at specific time intervals (*i.e.*, 3, 12, and 60 mo) by a variety of examinations (*e.g.*, coronary angiography, right heart catheterization, biplane left ventriculography, electrocardiogram at rest and exercise, echocardiography, late potential, heart rate variability and 24-h Holter electrocardiogram). The authors reported significant improvements as regards LV performance, quality of life and mortality in their 5-year data.

But there were also some studies (not few) that challenged these optimistic findings. In some cases, contradictory results of similar studies were revealed

Table 1 Hallmark clinical trials

Study name	Clinical trials (gov ID)	Principal investigator	No. of included patients
BOOST ^[10,18,19]	NCT00264316	Stefan Janssens	Treated (<i>n</i> = 30) Control (<i>n</i> = 30)
REPAIR-AMI ^[17,20,21]	NCT00279175	Andreas Zeiher	Treated (<i>n</i> = 101) Control (<i>n</i> = 103)
ASTAMI ^[23]	NCT00199823	Ketil Lunde	Treated (<i>n</i> = 50) Control (<i>n</i> = 50)
BALANCE ^[22]	-	Bodo-Eckehard Strauer	Treated (<i>n</i> = 62) Control (<i>n</i> = 62)
SWISS-AMI ^[24]	NCT00355186	Roberto Corti	Treated (<i>n</i> = 133) Control (<i>n</i> = 67)
TIME ^[25]	NCT00684021	Robert Simari	Treated (<i>n</i> = 79) Control (<i>n</i> = 41)
Late TIME ^[26]	NCT00684060	Robert Simari	Treated (<i>n</i> = 57) Control (<i>n</i> = 29)

simultaneously to the scientific community; this is the case of two well-known studies - REPAIR-AMI and ASTAMI respectively, which were published in the same issue of *The New England Journal of Medicine* in 2006^[17,23]. As opposed to REPAIR-AMI, the trial conducted by the Norwegian group reported no changes in LVEF, LV volumes or infarct size assessed at 6 mo by SPECT, echocardiography and CMR in 97 patients treated with intracoronary BMMNCs versus placebo a median of 6 d post AMI.

The pile of negative findings expanded based on the results of 3 other studies - namely SWISS-AMI^[24], TIME^[25] and Late TIME^[26] - thoroughly analyzed by Simari and colleagues in a paper on behalf of Cardiovascular Cell Therapy Research Network^[27]. The Cardiovascular Cell Therapy Research Network (CCTRN) was intended to enable cell based therapies in the United States^[28]; in this regard, CCTRN sponsored the TIME and LateTIME trials which aimed to evaluate the influence of BMMNCs delivery timing on LV function. The 3 studies mentioned above shared some similar characteristics, but differed in some other aspects. All were prospective, randomized, controlled trials designed to identify moderate to large placebo-adjusted LVEF improvements (from 3.5% to 5%) as assessed by CMR 4 or 6 mo after PCI. Cell dose and delivery were the same in each of the 3 studies - that was the intracoronary stop-flow technique described in the early 2000s^[7], but cell handling varied: It was manual Ficoll processing in SWISS-AMI, while the investigators of the CCTRN studies went for automated Ficoll processing. The authors reported no benefit of intracoronary administration of BMMNCs related to LV function irrespective of the timing of delivery. But why apparently similar studies led to contradictory results? These conflicting outcomes have been debated - and to some extent explained - by a series of experts in the field^[14,29,30].

A direct comparative analysis of methodology used in REPAIR-AMI^[17] and ASTAMI^[23] trials have revealed that seemingly minor changes in BMMNCs isolation and preservation protocols may have a major impact

on functional activity of isolated cells, consequently affecting the clinical outcome. Seeger *et al.*^[29] collected bone marrow from healthy volunteers or patients with angiographically confirmed coronary artery disease. Equal aliquots from the same bone marrow aspirate were manipulated accordingly to either REPAIR-AMI (density gradient centrifugation using Ficoll, followed by overnight incubation in *ex-vivo* 10 medium + 20% autologous serum at room temperature), or ASTAMI (density gradient centrifugation using Lymphoprep, followed by overnight incubation in 0.9% NaCl + 20% heparin-plasma at 4 °C) protocol. Obtained BMMNCs were subsequently tested for various parameters of phenotype and function, with quite divergent results. REPAIR-AMI isolation protocol generated a superior number of total BMMNCs, but also more haematopoietic and mesenchymal SCs as compared to ASTAMI. Furthermore, cells isolated and stored according to German study yielded better results in terms of proliferative capacity, ability to migrate to the chemoattractant SDF-1 and improvement in blood flow in a mouse model of hind-limb ischaemia.

Moreover, there is a substantial individual variability related to quantitative but also qualitative changes of adult bone marrow SCs with age, cardiovascular risk factors and associated comorbidities, decreasing the efficiency of cell therapy particularly in patients who need it the most^[31-35]. Studies have shown that young age and a superior number of CD34⁺ cells were independent predictors for treatment response to cell therapy, demonstrating the importance of patient's cell product^[36,37].

Additionally, the natural history of AMI has an unpredictable course modulated by upregulation and downregulation of a wide array of cytokines, growth and inflammatory factors. In specific subgroups of patients this changeable biological milieu could blur and/or make it difficult to distinguish a cell-based specific efficacy signal.

Some other potentially incriminated factors associated to result variability could be related to different times

between AMI and SCs delivery or to variability methods for the assessment of ventricular function and perfusion (ventriculography, echo-cardiography, CMR, SPECT).

META-ANALYSIS

Because of low sample size and small effects, individual studies were underpowered to identify significant differences in major adverse clinical events between SC therapy and control group. Therefore, new approaches were needed. In hope of obtaining clear answers regarding the effectiveness of SC therapy, several meta-analysis were carried-out since 2006, but the controversies continued^[13,36,38-52]. Extensive or less-extensive analysis were completed on different number of RCT (5-43) including different number of patients (482-2732 patients)^[38,53,54]. Subgroup analysis were performed based on different parameters such as baseline LVEF, timing of SCs infusion from onset of AMI, the dose of BMMNCs infused and patients age. Although earlier meta-analysis reported that intracoronary BMMNCs infusion is associated with significant improvements of LV function and remodeling particularly in younger patients and patients with a more severely depressed LVEF at baseline^[13,36,45], recent analysis revealed that intracoronary cell therapy provided only modest^[53,54] or no benefit in terms of clinical events or changes in LV function^[52].

But then, why such discrepancy even between meta-analysis reports? This was the theme of 2 very recent reviews published by well-known experts in the field^[55,56]. As one would expect, the first variation factor to point the finger to is related to differences in the methodology used in conducting systematic reviews. All meta-analyses except the one reporting negative findings relied on published summary results from multiple trials, while the latter was based on individual patient data (IPD) collected directly from the researchers responsible for each study and further centrally re-analyzed. The 2 methodologies varied in data collection, data checking and data analysis. Although ACCRUE (Meta-Analysis of Cell-Based Cardiac Studies; NCT01098591) database comprised a pool of 1252 IPDs from 12 randomized studies in AMI settings, it included only about 60% of the available published trials, as a result raising concern for potential bias. Of course, there are some other disparity factors involved, such as insufficient power of included studies, patients' heterogeneity and statistical heterogeneity^[55,56].

In view of presented data, one can only state that meta-analyses failed as well to clarify whether or not SC therapy improves heart function and/or mortality in AMI patients. What is more, meta-analyses are not surrogates for large phase III RCTs. Consequently, the scientific community is eagerly waiting for the ongoing BAMi trial to provide a more conclusive answer as regards the efficacy of bone marrow cell therapy in AMI settings. BAMi (the Effect of Intracoronary Reinfusion of Bone Marrow-Derived Mononuclear Cells on All Cause Mortality in AMI; NCT01569178) is the largest and most aspiring trial to date, funded by the European Commission

Seventh Framework Programme. It currently involves 19 partners planning to include 3000 patients from 10 European countries. The study aims to standardize methods of bone marrow cell collection, handling and delivery, as well as to test if the product and delivery method can lead to a 25% reduction in mortality.

PRESENT AND FUTURE STRATEGIES TO IMPROVE BONE MARROW SCs REGENERATIVE POTENTIAL

Since BMMNCs yielded only modest improvements regarding LV function recovery after AMI, selected bone marrow SCs populations have been tested in clinical settings. Trials involving CD34⁺/133⁺ progenitor cells^[57-61] or mesenchymal stem cells (MSCs)^[62-65] had encouraging results, but none of these tested cells haven't been clearly demonstrated to yield superior outcomes.

Of course, strategies to increase the number and potency of low-abundance progenitor cells in bone marrow cells (e.g., MSCs, CD34⁺/CD133⁺ cells) are needed. While in animal models a variety of genetic and nongenetic approaches aiming to improve therapeutic efficacy of transplanted cells have been tested, there is still a long road till translation into clinical settings. Some of the genetic strategies include enhancement of survival, proliferation and differentiation capacity, as well as boost of paracrine factors synthesis. Nongenetic procedures in essence comprise preconditioning with various factors (physical factors, drugs, cytokines and growth factors), 3D aggregate formation or hydrogel encapsulation and coculture with other types of SCs (e.g., cardiac SCs)^[66,67].

In addition, unlike in chronic ischemic disease, strategies to improve bone marrow SCs regenerative potential in acute settings are limited by the relatively short window of opportunity.

DRAWBACKS

Most important drawbacks and limitations of BMMNCs in AMI settings are related to reduced regenerative potential of transplanted cells; therefore, finding strategies to intensify their survival, proliferation and differentiation potential is a perpetual quest. But aside from these methodological features discussed in previous chapter, we would like to bring your attention to another issue, namely scientific inaccuracy. Unfortunately, SC research has not been avoided by scandals related to "scientific misconduct" in the field. It is the case of studies conducted by the German scientist Bodo-Eckehard Strauer. His papers have been comprehensively analyzed by Francis *et al.*^[68] who identified and exposed a series of discrepancies and contradictions such as number of patients receiving cells, baseline EF comparability and cell preparation. Although none of Strauer's studies have been retracted, their results cannot be trusted any more. Nevertheless, we chose to include them in our review in order to provide the reader

with an accurate depiction of SC therapy development in AMI settings, since the German investigator conducted not only the pioneering research in this area, but also one of the largest and most promising trials in the field. Besides affecting the credibility of the researchers, these inconsistencies may negatively influence the patients' decision when considering enrollment in a SC-based clinical trial.

PERSPECTIVES AND RECOMMENDATIONS

Predicting who will benefit and who will not from SC therapy is not currently possible, although efforts are being made in this direction^[37]. In the era of Precision Medicine Initiative^[69,70], being able to discriminate responders from nonresponders could be the first step toward tailored cell therapy. Prediction models for responder identification based on individual's characteristics are mandatory, in order that every single patient gets optimal treatment according to his individual variations in genes, environment and lifestyle.

Investigators of future trials should carefully choose hard clinically meaningful end points not limited to one effect, but rather reflecting different categories of consequences, such as structural evaluations of the heart, cardiovascular physiological measurements, biomarkers (including transcriptomic-based biomarkers), functional capacity and quality of life^[71].

The European Society of Cardiology Working Group Cellular Biology of the Heart has recently provided a series of recommendations on how to improve the therapeutic application of cell-based therapies for cardiac regeneration and repair^[72]. Accordingly, upcoming studies should be designed to address precise hypotheses on delivery types and mechanisms of efficiency, rather than safety and efficacy endpoints only; comparison of different cell types, or a combination of cell types in RCTs should be completed; in-depth cell characterization - including cell function should be done in every clinical trial; also, strategies to boost both cellular and paracrine effects should be developed.

CONCLUSION

A substantial knowledge has been gained in the past 15 years since the first bone marrow SCs transplantation have been performed in a patient with AMI, but there are a lot of challenges to be faced until this therapy will gain a definitive place in clinical arena.

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Next-generation sequencing traces human induced pluripotent stem cell lines clonally generated from heterogeneous cancer tissue

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Data sharing statement: The next-generation sequencing data in this study will be available to the public through the DDBJ Sequence Read Archive (DRA).

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Abstract

AIM

To investigate genotype variation among induced pluripotent stem cell (iPSC) lines that were clonally generated from heterogeneous colon cancer tissues using next-generation sequencing.

METHODS

Human iPSC lines were clonally established by selecting independent single colonies expanded from heterogeneous primary cells of S-shaped colon cancer tissues by retroviral gene transfer (*OCT3/4*, *SOX2*, and *KLF4*). The ten iPSC lines, their starting cancer tissues, and the matched adjacent non-cancerous tissues were analyzed using next-generation sequencing and bioinformatics analysis using the human reference genome hg19. Non-synonymous single-nucleotide variants (SNVs) (missense, nonsense,

and read-through) were identified within the target region of 612 genes related to cancer and the human kinome. All SNVs were annotated using dbSNP135, CCDS, RefSeq, GENCODE, and 1000 Genomes. The SNVs of the iPSC lines were compared with the genotypes of the cancerous and non-cancerous tissues. The putative genotypes were validated using allelic depth and genotype quality. For final confirmation, mutated genotypes were manually curated using the Integrative Genomics Viewer.

RESULTS

In eight of the ten iPSC lines, one or two non-synonymous SNVs in *EIF2AK2*, *TTN*, *ULK4*, *TSSK1B*, *FLT4*, *STK19*, *STK31*, *TRRAP*, *WNK1*, *PLK1* or *PIK3R5* were identified as novel SNVs and were not identical to the genotypes found in the cancer and non-cancerous tissues. This result suggests that the SNVs were *de novo* or pre-existing mutations that originated from minor populations, such as multifocal pre-cancer (stem) cells or pre-metastatic cancer cells from multiple, different clonal evolutions, present within the heterogeneous cancer tissue. The genotypes of all ten iPSC lines were different from the mutated *ERBB2* and *MKNK2* genotypes of the cancer tissues and were identical to those of the non-cancerous tissues and that found in the human reference genome hg19. Furthermore, two of the ten iPSC lines did not have any confirmed mutated genotypes, despite being derived from cancerous tissue. These results suggest that the traceability and preference of the starting single cells being derived from pre-cancer (stem) cells, stroma cells such as cancer-associated fibroblasts, and immune cells that co-existed in the tissues along with the mature cancer cells.

CONCLUSION

The genotypes of iPSC lines derived from heterogeneous cancer tissues can provide information on the type of starting cell that the iPSC line was generated from.

Key words: Colon cancer; Next-generation sequencing; Single-nucleotide variant; Genotype; Heterogeneous cancer tissue; Cancer associated fibroblast; Pre-cancer cell; Induced pluripotent stem cell; Single cell; Clonal evolution

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Core tip: Ten induced pluripotent stem cell (iPSC) lines were clonally generated from heterogeneous colon cancer tissues and analyzed with next-generation sequencing. Non-synonymous single-nucleotide variants (SNVs) of the iPSC lines were not identical to the genotypes of the cancer tissues. The SNVs were *de novo* or pre-existing mutations that originated from a minor population within the cancer tissue. Meanwhile, the genotypes of the iPSC lines were not mutated genotypes of the cancer tissues, suggesting that the starting cells for the iPSC lines were not mature cancer cells. Thus, the genotypes of iPSC lines can be used to trace the genomic origins of single

cells within heterogeneous cancer tissue.

Ishikawa T. Next-generation sequencing traces human induced pluripotent stem cell lines clonally generated from heterogeneous cancer tissue. *World J Stem Cells* 2017; 9(5): 77-88 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v9/i5/77.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v9.i5.77>

INTRODUCTION

Gene transfer of *OCT3/4*, *SOX2*, *KLF4*, and *c-Myc* to somatic cells generates human induced pluripotent stem cells (iPSCs)^[1-3] although *c-MYC* is not required for iPSC generation^[4]. Human iPSCs are indistinguishable from human embryonic stem cells (ESCs) in terms of their long-term self-renewal ability and their *in vivo* pluripotency^[3,5]. The starting cells for iPSC generation should be appropriately chosen to generate normal or aberrant iPSC lines for the purpose of regenerative medicine or cancer research/therapy. Human iPSC lines for regenerative medicine would be ideally generated from normal neonatal tissues^[3] that are typically free of postnatal aberrant mutations and epigenetic changes. Human iPSCs (or iPSC-like cells) have also been generated from cancer cell lines^[6,7], the somatic cells from familial cancer patients^[8,9], and pancreatic ductal adenocarcinomas^[10]. For cancer research/therapy, it is of great interest to generate iPSCs from heterogeneous cancer tissues. In our recent study^[11], human iPSC lines were clonally generated from a heterogeneous mixture of primary cells derived from gastric tissues or colon cancer tissues and were subjected to microarray gene expression analysis. The resultant iPSC lines expressed all ESC-enriched genes including *POU5F1*, *SOX2* and *NANOG* that are essential for self-renewal ability and pluripotency^[5,12] at a level equivalent to those of the typical human iPSC line (201B7)^[11]. Genome-wide gene expression patterns were used to categorize the reference iPSC line 201B7 and the iPSC lines derived from distinct cancer tissues into three different groups. The gene expression profiles of these iPSC lines demonstrated differences derived from their distinct starting tissues and similarity and heterogeneity derived from their common starting heterogeneous tissues. More recently, it was reported that reference component analysis (RCA), an algorithm that substantially improves clustering accuracy, was developed to robustly cluster single-cell transcriptomes^[13]. The RCA of single-cell transcriptomes elucidated cellular heterogeneity in human colorectal cancer^[13].

In this study, iPSC technology and next-generation sequencing were used to resolve genotype variation among single cells within a heterogeneous cancer tissue. The genomic DNA of ten iPSC lines that were clonally generated from human colon cancer tissue was analyzed and compared with the genomic DNA from their cancer tissue of origin and matched adjacent non-cancerous

tissue.

MATERIALS AND METHODS

Tissues derived from a single colon cancer patient

This study was conducted with the approval of the Institutional Review Boards of the National Cancer Center of Japan and the Japanese Collection of Research Bioresources (JCRB), National Institutes of Biomedical Innovation, Health and Nutrition. Written informed consent from a single donor was obtained for the use of the tissues for research. The anonymous remnant non-cancerous and cancerous tissues were provided by the JCRB Tissue Bank. The tissues were derived from the surgical waste material from an operation performed on a 55-year-old Japanese male S-shaped colon cancer patient.

Primary cell culture from cancer tissues

Heterogeneous primary cell culture from the colon cancer tissues was prepared as previously described^[11]. Briefly, the tissues were washed with Hank's balanced salt solution (HBSS) and minced into pieces with scissors. The pieces were further washed with HBSS. DMEM with collagenase was added to the tissue precipitates and mixed at 37 °C for 1 h on a shaker. After washing with DMEM, cells were seeded on collagen-coated dishes and cultured in DMEM supplemented with 10% FBS.

Generation of human iPSC lines

The study was approved by the Institutional Recombinant DNA Advisory Committee. Heterogeneous primary cells from the cancer tissue were cultured for 24 h at approximately 5%-10% confluency and then incubated with a pantropic retrovirus vector solution (*OCT3/4*, *KLF4*, and *SOX2*) at 37 °C for an additional 24 h. The vector solution was prepared as previously described^[14]. Mitomycin C-treated mouse embryonic fibroblasts (MEFs, ReproCell) were seeded and co-cultured with the primary cells following the retroviral infection. The culture medium was replaced with MEF-conditioned ESC medium every 3 d until the cell layer was fully confluent and then further refreshed with mTeSR1 medium (STEMCELL Technologies) every day. Each independent colony was isolated from the culture using forceps under a microscope. The independent iPSC lines were sub-cultured with MEF in gelatin-coated 24-well plates.

Expansion and passage culture of human iPSC lines

Human iPSC lines were cultured with the MEFs in primate ESC, ReproStem (ReproCell) or mTeSR1 medium in gelatin-coated dishes^[11]. The expanded iPSC lines were treated with a dissociation solution (ReproCell) or 0.25% trypsin-EDTA (Gibco) and passaged in media supplemented with 10-20 µmol/L Y-27632 to avoid cell death^[3]. Independent iPSC lines were passaged from the 24-well plates into 6-well plates, further expanded into 100-mm dishes, and minimally passaged in 100-mm dishes under similar

culture conditions. Each genomic DNA sample was prepared from independent iPSC lines.

Real-time RT-PCR analysis

Total RNA was prepared using the miRNeasy Mini Kit (Qiagen). Reverse transcription of the RNA was carried out using an iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad). Quantitative PCR was carried out with an SsoAdvanced Universal SYBR® Green Supermix using the CFX96 Real-Time PCR Detection System (Bio-Rad). PCR primer sets for *OCT3/4*, *SOX2*, *NANOG*, *ZFP42*, and *GAPDH* are listed in Supplemental Table 1. PCR data were analyzed using CFX Manager Software (Bio-Rad). PCR data from the iPSC 201B7^[11] RNA were used as a positive control, and PCR data from cancer tissue-derived iPSC lines are presented as quantification cycle (Cq) values.

Target selection and sequencing

Target sequencing was conducted for twelve DNA samples from the cancer tissues, the non-cancerous tissues, and the ten iPSC lines. Genomic DNA was extracted from each of twelve samples using the DNeasy Blood AND Tissue Kit (Qiagen), sheared into approximately 150-bp fragments, and used to make a library for multiplexed paired-end sequencing with the SureSelect^{XT} Reagent Kit (Agilent Technologies). The constructed library was hybridized to biotinylated cRNA oligonucleotide baits from the SureSelect^{XT} Human Kinome Kit (Agilent Technologies) for target enrichment. Targeted sequences were purified by magnetic beads, amplified, and sequenced on an Illumina HiSeq2000 platform in a paired-end 101 bp configuration.

Mapping and single-nucleotide variant calling

Adapter sequences were removed by cutadapt (v1.2.1). After quality control, reads were mapped to the human reference genome hg19 using BWA (ver.0.6.2). Mapping results were corrected using Picard (ver.1.73) for removing duplicates and GATK (ver.1.5-32) for local alignment and base quality score recalibration. Single-nucleotide variant (SNV) calls were performed with multi-sample calling using GATK (UnifiedGenotyper) and filtered to coordinates with a variant call quality score ≥ 30 and a depth ≥ 8 . SNVs were further classified based on their predicted functions of missense, nonsense or read-through. For final confirmation, SNVs were manually curated using the Integrative Genomics Viewer. Annotations of SNVs were based on dbSNP135, CCDS (NCBI release 20111122), RefSeq (UCSC Genome Browser, dumped 20111122), GENCODE (UCSC Genome Browser, ver. 7), and 1000 Genomes (release 2011011) sequences.

RESULTS

Human iPSC lines derived from colon cancer tissues

The human iPSC lines CC1-1, CC1-2, CC1-7, CC1-8, CC1-9, CC1-11, CC1-12, CC1-17, CC1-18, and CC1-25

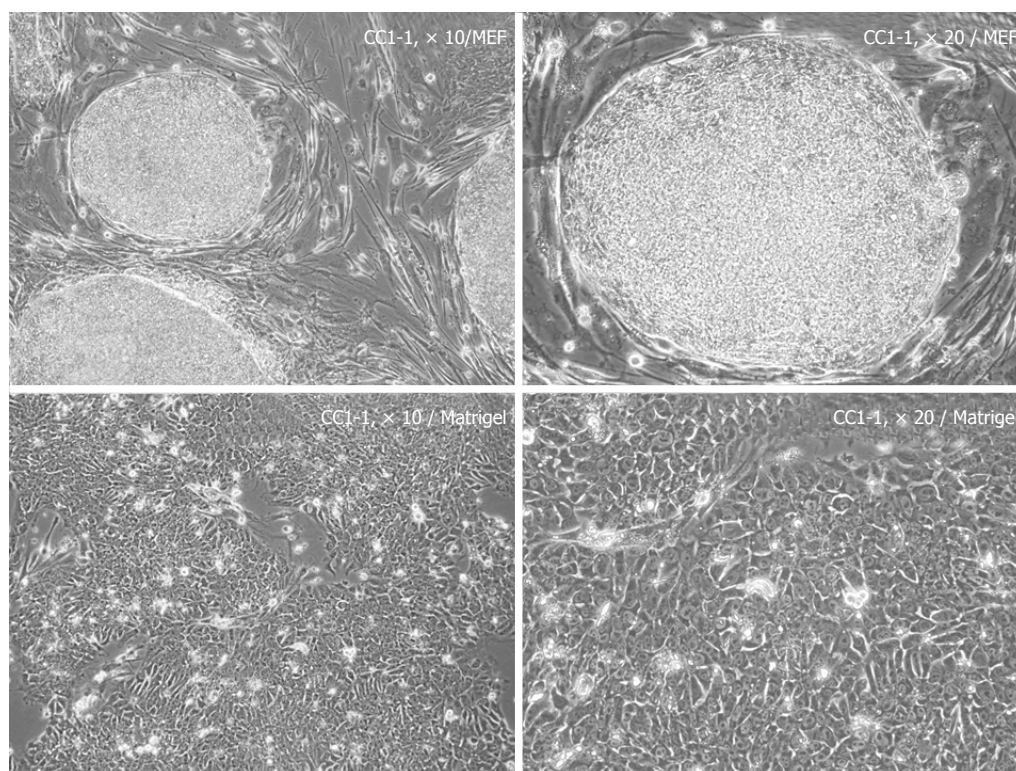


Figure 1 Phase contrast micrographs of colon cancer tissue-derived induced pluripotent stem cells. The human iPSC line CC1-1 was expanded with mitomycin C-treated mouse embryonic fibroblasts in gelatin-coated dishes (upper left panel: $\times 10$, upper right panel: $\times 20$) and cultured with feeder-free mTeSR1 medium in BD Matrigel™-coated dishes (lower left panel: $\times 10$, lower right panel: $\times 20$). iPSC: Induced pluripotent stem cell; MEF: Mouse embryonic fibroblast.

were donally generated from heterogeneous primary cells cultured from colon cancer tissue. The iPSC lines were expanded serially with MEFs in gelatin-coated dishes. The cancer tissue-derived iPSCs were indistinguishable in morphology from typical (fibroblast-derived) human iPSCs under conventional culture with MEFs (upper panels in Figure 1 and Supplemental Figure 1). The human iPSCs formed colonies consisting of very small cells and were efficiently passaged at a high recovery ratio with the addition of 10–20 $\mu\text{mol/L}$ Y-27632 to the cell culture medium. Human iPSCs were also cultured with feeder-free mTeSR1 medium in BD Matrigel™-coated 100-mm dishes and showed a high nucleus-to-cytoplasm ratio (lower panels in Figure 1 and Supplemental Figure 1).

Expression of human ESC-essential genes

ESC-essential gene expression of the cancer tissue-derived iPSC lines was quantitatively analyzed by real-time RT-PCR. All ten iPSC lines expressed *POU5F1*, *SOX2*, and *NANOG*, which are essential for self-renewal and pluripotency, at a level equivalent to those of the reference iPSC line (Supplemental Table 2). The study results support previously published microarray data showing that cancer tissue-derived iPSCs equally express ESC-enriched genes^[11].

Next-generation sequencing

The target region (SureSelect Human Kinome) in genomic DNA samples from the ten iPSC lines, their starting cancer tissues, and the matched adjacent non-

cancerous tissues was analyzed using next-generation sequencing. The target region of approximately 3.2 Mb covers the genome of the coding region of all known protein kinase genes and selected oncogenes and tumor suppressor genes, for a total of 612 genes (Supplemental Table 3). The original reads (2.6–4.0 Gb of sequence) were obtained from each genomic DNA sample by sequencing (Table 1). The modified reads were generated from the original reads (Table 2). The results of the mapped reads, the sequencing depth, and target capture are summarized in Tables 3–5. The average depth on the target region ranged from 317 to 496. More than 99.76% of the target region was covered with at least $8 \times$ depth for high-quality genotype calls (variant call quality score ≥ 30).

Non-synonymous SNVs compared with hg19

After sequencing, the reads underwent bioinformatics analysis (Figure 2). Through comparison with the human reference genome hg19, the non-synonymous SNVs (missense, nonsense or read-through) were called on the target region (on and near DNA target enrichment baits) of twelve samples (the ten iPSC lines, their starting cancer tissues, and the matched adjacent non-cancerous tissues). Of the resulting 378 non-synonymous SNVs (Supplemental Table 4), 50 were novel SNVs (not reported in dbSNP135 or 1000 Genomes, Supplemental Table 5).

Confirmed genotypes of the twelve samples

Of the 378 non-synonymous SNVs from the twelve

Table 1 Read number (original)

Sample	Original		
	No. of reads	Read length (b)	No. of bases (Gb)
NCC1	18260718	101	3.7
	18260718	101	
CC1	16706190	101	3.4
	16706190	101	
CC1-1	13045740	101	2.6
	13045740	101	
CC1-2	17725772	101	3.6
	17725772	101	
CC1-7	14780507	101	3.0
	14780507	101	
CC1-8	17311972	101	3.5
	17311972	101	
CC1-9	16664067	101	3.4
	16664067	101	
CC1-11	15455638	101	3.1
	15455638	101	
CC1-12	15391361	101	3.1
	15391361	101	
CC1-17	19009957	101	3.8
	19009957	101	
CC1-18	19746313	101	4.0
	19746313	101	
CC1-25	15492560	101	3.1
	15492560	101	

NCC1: The matched adjacent non-cancerous tissue; CC1: The starting cancer tissue of the induced pluripotent stem cell lines; CC1-1 to CC1-25: Each induced pluripotent stem cell line.

samples, 40 were distinct heteroallelic genotypes and included known SNVs in the 612 sequenced gene target region. Supplemental Table 6 lists the forty SNVs that were distinct among the human iPSC lines, their starting cancer tissue, and the matched adjacent non-cancerous tissues. The allelic depth and genotype quality of thirteen of the forty SNVs were validated and manually curated using the Integrative Genomics Viewer (Figure 2).

Mutated genotypes of cancer tissue-derived iPSC lines

The chromosome number, genome position, novelty, gene symbol, and mutation type of the thirteen confirmed SNVs are shown in Table 6; the allelic depth is shown in Table 7; and the genotype is shown in Table 8. The respective SNVs of the ten iPSC lines were compared to those of their starting cancer tissue and the matched non-cancerous tissue. The genotypes, which showed nonsense or missense mutations in *EIF2AK2*, *TTN*, *ULK4*, *TSSK1B*, *FLT4*, *STK19*, *STK31*, *TRRAP*, *WNK1*, *PLK1*, or *PIK3R5* (Table 6), of the iPSC lines were different from that of the non-cancerous tissue sample (Table 8). Nevertheless, the genotypes of the iPSC samples were also different from that of the starting cancer tissue sample. The heteroallelic read sequences of *ULK4*, *TRRAP*, and *WNK1* of the starting cancer tissue sample consisted of 247|2 of A|C, 240|1 of G|C, and 246|2 of C|T, respectively (Table 7). Although the major read sequences indicated the genotypes of the non-cancerous tissues, the minor reads indicated missense mutations. The potential heteroallelic genotypes were identical

Table 2 Read number (modified)

Sample	Modified ¹		
	No. of reads	Read length (b)	Ratio (%) (Mod/Ori)
NCC1	18146940	101 ²	99.38
	18146940	101 ²	99.38
CC1	16597436	101 ²	99.35
	16597436	101 ²	99.35
CC1-1	12942132	101 ²	99.21
	12942132	101 ²	99.21
CC1-2	17614866	101 ²	99.37
	17614866	101 ²	99.37
CC1-7	14687008	101 ²	99.37
	14687008	101 ²	99.37
CC1-8	17180329	101 ²	99.24
	17180329	101 ²	99.24
CC1-9	16545785	101 ²	99.29
	16545785	101 ²	99.29
CC1-11	15346749	101 ²	99.30
	15346749	101 ²	99.30
CC1-12	15281269	101 ²	99.28
	15281269	101 ²	99.28
CC1-17	18880292	101 ²	99.32
	18880292	101 ²	99.32
CC1-18	19618808	101 ²	99.35
	19618808	101 ²	99.35
CC1-25	15378555	101 ²	99.26
	15378555	101 ²	99.26

¹Modified read file is a data set from the original read file with the adapter sequences and low-quality bases removed; ²Therefore, there were reads shorter than the number indicated by the read length (b) in a portion of the modified read file. NCC1: The matched adjacent non-cancerous tissue; CC1: The starting cancer tissue of the induced pluripotent stem cell lines; CC1-1 to CC1-25: Each induced pluripotent stem cell line.

to the mutated genotypes of the CC1-25, CC1-12, and CC1-8 iPSC lines. Meanwhile, the genotypes of all ten of the iPSC lines were different from the mutated genotypes in *ERBB2* and *MKNK2* of the cancer tissues and were identical to those of the non-cancerous tissues and human reference genome hg19 (Table 8). Thus, all analyzed iPSC lines were preferentially generated from starting cells without mutations in *ERBB2* and *MKNK2*, except for those generated from mature cancer cells. Furthermore, the iPSC lines CC1-7 and CC1-17 did not have any confirmed mutated genotypes despite originating from the cancer tissue.

DISCUSSION

The ten iPSC lines were clonally generated from a heterogeneous mixture of primary cells derived from the colon cancer tissue of a single patient. The genomes of the ten iPSC lines were analyzed using next-generation sequencing. The genomes of the starting cancer tissue and matched adjacent non-cancerous tissue from the same donor were also analyzed. The target region for analysis was the human kinome and cancer-related genes that are typically mutated in human tumors. A total of 378 non-synonymous SNVs identified from samples of the ten iPSC lines and the cancerous and non-cancerous tissues were identified by comparing the sequence reads

Table 3 Mapped reads

	NCC1	CC1	CC1-1	CC1-2	CC1-7	CC1-8	CC1-9	CC1-11	CC1-12	CC1-17	CC1-18	CC1-25
No. of total reads ①	36293880	33194872	25884264	35229732	29374016	34360658	33091570	30693498	30562538	37760584	39237616	30757110
No. of mapped reads ② (③+ ④ + ⑤)	36210841	33066194	25545096	34845596	28911555	33842665	32976780	29898986	30464308	36670526	38078508	30173511
No. of mapped reads with Paired-End ③	26935180	26333748	21884450	25981140	21498822	26807330	26704280	25386830	25103580	29513694	30869186	21622036
No. of mapped reads with Single-End ④	15741	24050	14051	16508	12336	17984	14651	27429	14354	27336	25456	19687
No. of discarded reads ¹ ⑤	9259920	6708396	3646595	8847948	7400397	7017351	6257849	4484727	5346374	7129496	7183866	8531788
No. of unmapped reads (① - ②)	83039	128678	339168	384136	462461	517993	114790	794512	98230	1090058	1159108	583599
No. of effective reads (③ + ④)	26950921	26357798	21898501	25997648	21511158	26825314	26718931	25414259	25117934	29541030	30894642	21641723

¹Discarded reads were as follows: Reads mapped to chromosomes other than the target; Reads where each paired-end is mapped to a different chromosome; Reads not used for single-nucleotide variant/InDel detection such as PCR duplicates. Each number of ② consists of each total number of "③ plus ④ plus ⑤"; "① - ②" means "each number of ① minus each number of ②"; "③ + ④" means "each number of ③ plus each number of ④". NCC1: The matched adjacent non-cancerous tissue; CC1: The starting cancer tissue of the iPSC lines; CC1-1 to CC1-25: Each induced pluripotent stem cell line.

Table 4 Sequence depth

Sample	Theoretical depth ¹		Observed depth ²	
	Total bases	Depth ³	Effective bases on target (Mb)	Average depth on target
NCC1	3689	1173.31	1399	445.06
CC1	3375	1073.43	1084	344.89
CC1-1	2635	838.23	999	317.89
CC1-2	3581	1138.94	1279	406.72
CC1-7	2986	949.69	1195	380.13
CC1-8	3497	1112.35	1352	430.01
CC1-9	3366	1070.72	1355	431.06
CC1-11	3122	993.07	1077	342.50
CC1-12	3109	988.94	1359	432.12
CC1-17	3840	1221.45	1407	447.66
CC1-18	3989	1268.76	1559	495.99
CC1-25	3129	995.45	1083	344.37

¹Theoretical depth calculated from the total number of bases obtained by DNA sequencing; ²Observed depth used for single-nucleotide variant/InDel identification; ³Theoretical depth [Total Bases (Mb)]/[Target region (Mb)]. Target region: SureSelect Human Kinome Kit (approximately 3.1 Mb). NCC1: The matched adjacent non-cancerous tissue; CC1: The starting cancer tissue of the induced pluripotent stem cell lines; CC1-1 to CC1-25: Each induced pluripotent stem cell line.

to the human reference genome hg19. Most of the non-synonymous SNVs showed the genotype of the non-cancerous tissue, suggesting their germline origin. The SNVs of the ten iPSC lines were compared with those of the cancerous and non-cancerous tissues. Forty of the SNVs were distinct genotypes among all twelve samples. Thirteen of the forty SNVs were confirmed using allelic depth, genotype quality, and the Integrative Genomics Viewer.

In eight of the ten iPSC lines, one or two novel, non-synonymous SNVs (heteroallelic missense or nonsense mutation) in *EIF2AK2*, *TTN*, *ULK4*, *TSSK1B*, *FLT4*, *STK19*, *STK31*, *TRRAP*, *WNK1*, *PLK1* or *PIK3R5* were identified as genotypes different from those of the non-cancerous tissue. Unexpectedly, all the SNVs were not identical to the genotypes found in the cancer tissues. Because of

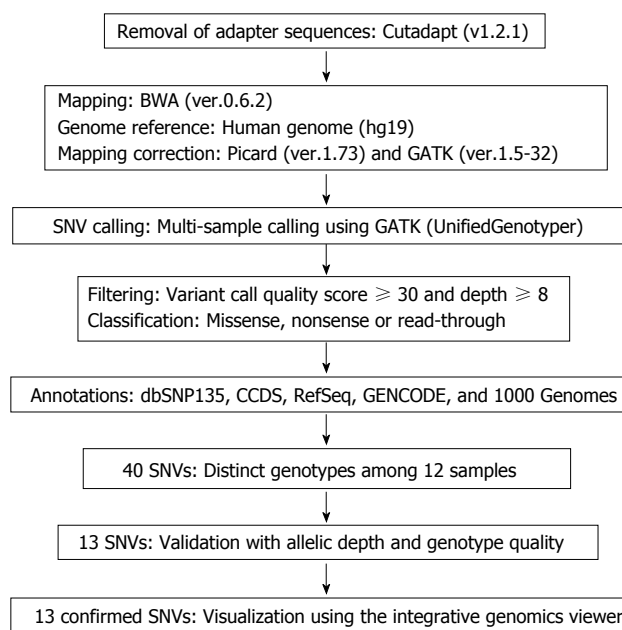
Pipeline of bioinformatics analysis

Figure 2 Pipeline of bioinformatics analysis following next-generation sequencing. The thirteen confirmed SNVs are shown in Tables 6-8. SNVs: Single-nucleotide variants.

minor read sequences, the potential genotype of *ULK4*, *TRRAP* or *WNK1* in the cancer tissues was implied. The sequences indicated a missense mutation in *ULK4*, *TRRAP* or *WNK1* identical to that found in the iPSC lines CC1-25, CC1-12, and CC1-8. Accordingly, there is a possibility that each iPSC line was generated from a starting cell from a minor cell population with a mutation in *ULK4*, *TRRAP*, or *WNK1* that was present within the cancer tissue. The minor read sequences could be confirmed by ultra-deep sequencing to support the potential heteroallelic genotypes. Interestingly, two iPSC lines CC1-7 and CC1-17 did not have any confirmed mutated genotypes despite originating from the cancer

Table 5 Target capture

	NCC1	CC1	CC1-1	CC1-2	CC1-7	CC1-8	CC1-9	CC1-11	CC1-12	CC1-17	CC1-18	CC1-25
Initial bases on target ①	3143812	3143812	3143812	3143812	3143812	3143812	3143812	3143812	3143812	3143812	3143812	3143812
Initial bases near target ②	3790645	3790645	3790645	3790645	3790645	3790645	3790645	3790645	3790645	3790645	3790645	3790645
Initial bases on or near target ③	6934457	6934457	6934457	6934457	6934457	6934457	6934457	6934457	6934457	6934457	6934457	6934457
Total effective reads ④	26950921	26357798	21898501	25997648	21511158	26825314	26718931	25414259	25117934	29541030	30894642	21641723
Total effective bases (Mb) ⑤	2663	2602	2157	2573	2125	2643	2637	2503	2480	2914	3047	2133
Read length mean (b)	98.91	98.83	98.58	99.02	98.84	98.61	98.77	98.58	98.79	98.74	98.69	98.65
Read length median (b)	101	101	101	101	101	101	101	101	101	101	101	101
Effective bases on target (Mb) ⑥	1399	1084	999	1279	1195	1352	1355	1077	1359	1407	1559	1083
Effective bases near target (Mb) ⑦	440	342	355	405	380	438	448	327	445	430	450	342
Effective bases on or near target (Mb) ⑧	1839	1426	1354	1683	1575	1790	1804	1403	1804	1837	2009	1425
Fraction of effective bases on target (%) (⑥/⑤)	52.54	41.67	46.32	49.70	56.25	51.14	51.38	43.01	54.78	48.30	51.18	50.75
Fraction of effective bases near target (%) (⑦/⑤)	16.52	13.14	16.45	15.73	17.88	16.56	17.00	13.05	17.96	14.75	14.77	16.05
Fraction of effective bases on or near target (%) (⑧/⑤)	69.06	54.82	62.78	65.43	74.12	67.70	68.38	56.06	72.74	63.05	65.95	66.80
Average sequencing depth on target (⑥/①)	445.06	344.89	317.89	406.72	380.13	430.01	431.06	342.50	432.12	447.66	495.99	344.37
Average sequencing depth near target (⑦/②)	116.05	90.22	93.63	106.76	100.21	115.48	118.31	86.16	117.51	113.43	118.68	90.33
Average sequencing depth on or near target (⑧/③)	265.21	205.68	195.30	242.75	227.11	258.07	260.10	202.38	260.14	264.96	289.74	205.50
Base covered on target ⑨	3143221	3143152	3142784	3143540	3143280	3143263	3143277	3142887	3143338	3143035	3143296	3142818
Coverage of target region (%) (⑨/①)	99.98	99.98	99.97	99.99	99.98	99.98	99.98	99.97	99.98	99.98	99.98	99.97
Base covered near target ⑩	3775671	3773869	3774076	3771915	3768892	3773823	3776942	3760218	3776060	3766215	3762031	3762823
Coverage of near target region (%) (⑩/②)	99.60	99.56	99.56	99.51	99.43	99.56	99.64	99.20	99.62	99.36	99.25	99.27
Fraction of target covered with at least 15 × (%)	99.72	99.62	99.59	99.69	99.65	99.68	99.70	99.55	99.69	99.60	99.68	99.58
Fraction of target covered with at least 8 × (%)	99.86	99.78	99.78	99.83	99.81	99.82	99.83	99.76	99.83	99.78	99.83	99.77
Fraction of target covered with at least 4 × (%)	99.93	99.89	99.89	99.90	99.90	99.90	99.91	99.87	99.92	99.89	99.91	99.89
Fraction of flanking region covered with at least 15 × (%)	86.88	84.24	87.52	85.84	84.62	87.92	89.85	79.85	90.17	84.50	80.72	82.36
Fraction of flanking region covered with at least 8 × (%)	94.32	93.13	94.66	93.66	92.95	94.70	95.77	89.93	95.87	92.55	89.96	91.39
Fraction of flanking region covered with at least 4 × (%)	97.85	97.33	97.91	97.50	97.15	97.87	98.27	95.76	98.32	96.86	95.71	96.37

The target region, as covered by the SureSelect Human Kinome Kit, was approx. 3.1 Mb. Near target region was 200 bases forward and backward of the target region. "⑥/⑤" means "each number of ⑥ divided by each number of ⑤"; "⑦/⑤" means "each number of ⑦ divided by each number of ⑤"; "⑧/⑤" means "each number of ⑧ divided by each number of ⑤"; "⑥/①" means "each number of ⑥ divided by each number of ①"; "⑦/②" means "each number of ⑦ divided by each number of ②"; "⑧/③" means "each number of ⑧ divided by each number of ③"; "⑨/①" means "each number of ⑨ divided by each number of ①"; "⑩/②" means "each number of ⑩ divided by each number of ②". NCC1: The matched adjacent non-cancerous tissue; CC1: The starting cancer tissue of the induced pluripotent stem cell lines; CC1-1 to CC1-25: Each induced pluripotent stem cell line.

tissues. Therefore, the two iPSC lines might be generated from non-cancerous cells such as pre-cancer (stem) cells and cancer-associated fibroblasts^[15,16].

The SNVs of the ten iPSC lines could be *de novo* or pre-existing mutations that originated from minor cell populations, such as multifocal cancer (stem) cells and pre-metastatic cancer cells, present within the

heterogeneous cancer tissue. Primary cancer tissues include multifocal pre-, mature and pre-metastatic cancer cells, so it makes sense that their genomes would be heterogeneous. The genotypes of pre-cancer (stem) cells would not be identical to those of germline or mature cancer cells, as colon cancer develops from an adenoma to carcinoma through the accumulation of a number

Table 6 Chromosome number, genome position, reference *vs* single-nucleotide variant, novelty *vs* dbSNP135, gene symbol, and mutation types of single-nucleotide variants

SNV No.	Chromosome No.	Genome position	Ref. SNV	Novel/known	Gene symbol	Mutation types
1	chr2	37336419	C T	Novel	<i>EIF2AK2</i>	Missense
2	chr2	179408086	A G	Novel	<i>TTN</i>	Missense
3	chr3	41705179	A C	Novel	<i>ULK4</i>	Missense
4	chr5	112769527	C T	Novel	<i>TSSK1B</i>	Missense
5	chr5	180048626	C T	Novel	<i>FLT4</i>	Missense
6	chr6	31947203	T C	Novel	<i>STK19</i>	Missense
7	chr7	23808650	G T	Novel	<i>STK31</i>	Missense
8	chr7	98490141	G C	Novel	<i>TRRAP</i>	Missense
9	chr12	1009680	C T	Novel	<i>WNK1</i>	Missense
10	chr16	23690401	C T	Novel	<i>PLK1</i>	Missense
11	chr17	8789811	G A	Novel	<i>PIK3R5</i>	Nonsense
12	chr17	37881392	A G	Novel	<i>ERBB2</i>	Missense
13	chr19	2046399	G A	Novel	<i>MKNK2</i>	Missense

Ref.: The allele of the human reference genome hg19; SNV: Single-nucleotide variant.

of genetic mutations and epigenetic aberration^[17]. It is likely that the genotypes of pre-metastatic cancer cells in multiple clonal evolutions would be different from those of non-metastatic cancer cells. Meanwhile, genotypes of major mature cancer cells would be identical to those of cancer tissues; therefore, it was expected that genotypes of cancer tissue-derived iPSC lines would be identical to those of their starting cancer tissues. It was reported that *ERBB2* mutations were persistent in 3.6% of patients with colorectal cancer^[18]. Indeed, a mutated genotype in *ERBB2* of the colon cancer tissues was also identified in this study.

Nevertheless, the genotypes of the ten iPSC lines were different from the mutated *ERBB2* and *MKNK2* genotypes in the cancer tissues and were identical to those of the non-cancerous tissues and the human reference genome hg19. This result suggests that the starting cells for the iPSC lines did not carry the mutations in *ERBB2* and *MKNK2* present in the cancer tissues. It is conceivable that the non-mutated genotypes of each iPSC line were identical to those of non-cancerous cells such as pre-cancer (stem) cells, stroma cells and immune cells that existed within the tissue. Each iPSC line was clonally established by selecting an independent single colony expanded from a putative single starting cell originating from heterogeneous cancer tissue. The genome sequence of each iPSC line was derived from its starting single cell. As a result, each iPSC line conserved the non-mutated *ERBB2* and *MKNK2* genotypes that originated from their respective starting single cells. Interestingly, all ten iPSC lines were not generated from cell populations containing either a mutated *ERBB2* and/or a mutated *MKNK2*. Thus, the genotypes of each iPSC line provide information on the genomic origin of the starting single cell derived from the heterogeneous cancer tissue.

Although the cause of the preference for the genomic origin of their starting cells was not clarified in this study, it seems that chemicals^[19], gene sets^[1,4], gene

transfer^[20,21], or inventive pre-culture^[22,23], in which the starting cells might be preferentially specified, can affect iPSC generation. Accordingly, materials and methods can be optimized to generate normal or aberrant iPSC lines for the purposes of regenerative medicine or cancer research/therapy. Cancer tissues comprise (pre-) cancer (stem) cells, pre-metastatic cancer cells, stromal cells (such as mesenchymal stem cells, cancer-associated fibroblasts^[15,16,24] and tumor endothelial cells) and immune cells (such as tumor-associated macrophages^[25], dendritic cells^[26] and tumor-infiltrating T cells^[23]). Therefore, such a cell-derived iPSC line might be useful for immune-cell therapy^[27] with cellular vaccines^[28], dendritic cells^[29-32] or tumor antigen-specific cytotoxic T cells^[23], in addition to the development of models of carcinogenesis^[33-35] and drug discovery tools^[36,37]. For the purposes of regenerative medicine, human iPSCs are ideally generated from normal neonatal tissues^[3,38-40] that are typically inexperienced of postnatal aberrant mutations or epigenetic changes. By contrast, aging and sun-exposed skin carries thousands of evolving clonal cells carrying cancer-causing mutations^[41,42]. Indeed, genetic mutations accumulate gradually over a lifetime, even in human somatic stem cells^[43]. For this reason, cell sources for iPSC generation should be selected based on the given field of research. Furthermore, iPSC lines with few or no mutations need to be established by the modification of existing methodology^[39,44,45], as cell lines with *de novo* mutations not originating from the starting cells are not desired^[46-50].

Nevertheless, cancer tissue-derived iPSCs might give rise to such *de novo* mutations, as their starting cells might have already suffered from an aberration (epigenetics or gene expression) associated with *de novo* mutations or cancer. Indeed, colon cancer tissue-derived iPSC lines exhibited unique gene expression profiles, with particular upregulation of *FAM19A5* and *SLC39A7*^[11], in comparison with those of the typical iPSC line 201B7^[1]. *FAM19A5* and *SLC39A7* were found to be expressed at lower levels in many human iPSC and ESC lines based on a free online expression atlas (Amazonia!, <http://amazonia.transcriptome.eu/search.php>)^[51]. *FAM19A5* was reported as a novel cholangiocarcinoma biomarker^[52], while *SLC39A7* is an intracellular zinc transporter and a hub for tyrosine kinase activation related to diseases such as cancer^[53]. The analysis of iPSC genomes might expose rare single cells, such as an authentic cancer stem cells present within cancer tissues. Thus, next-generation sequencing of heterogeneous cancer tissue-derived iPSC lines might reveal potential aberrations or changes originating from the cancer tissue.

In conclusion, the genotypes of iPSC lines can be used to trace the genotype of the original single cells derived from heterogeneous cancer tissues.

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Table 7 Allelic depth of single-nucleotide variants among the matched adjacent non-cancerous tissue, the starting cancer tissue, and the cancer tissue-derived induced pluripotent stem cell lines

SNV No.	Allelic depth of SNVs											
	NCC1	CC1	CC1-1	CC1-2	CC1-7	CC1-8	CC1-9	CC1-11	CC1-12	CC1-17	CC1-18	CC1-25
1	250 0	246 0	232 0	250 0	250 0	250 0	250 0	248 0	250 0	250 0	129 121	250 0
2	249 0	240 0	240 0	248 0	248 1	250 0	129 121	248 0	242 0	250 0	250 0	244 0
3	246 0	247 2	249 0	238 1	246 0	248 0	233 0	241 0	238 1	241 0	245 0	132 106
4	250 0	239 0	243 0	248 0	245 0	120 129	250 0	236 0	250 0	250 0	250 0	249 0
5	216 0	150 0	75 79	189 0	184 0	180 0	200 1	131 0	176 0	221 0	207 0	179 0
6	249 0	238 0	250 0	132 114	250 0	250 0	242 0	248 0	248 0	250 0	250 0	249 0
7	250 0	248 0	250 0	250 0	245 0	246 0	245 0	135 111	249 0	250 0	250 0	246 1
8	233 0	240 1	243 0	250 0	245 0	242 0	247 0	248 0	132 113	240 1	247 0	241 0
9	249 0	246 2	250 0	250 0	249 0	220 30	244 0	249 0	250 0	249 1	250 0	249 0
10	247 0	177 0	188 0	119 121	198 0	244 0	241 0	176 0	221 0	224 0	249 0	174 0
11	246 1	172 0	181 0	208 0	209 0	198 0	189 0	175 0	244 0	182 0	233 0	95 87
12	249 1	195 54	241 0	249 0	249 0	249 1	249 0	250 0	249 0	250 0	249 1	250 0
13	137 0	91 10	79 0	131 0	102 0	103 0	103 0	83 0	106 0	111 0	142 0	90 0

NCC1: The matched adjacent non-cancerous tissue; CC1: The starting cancer tissue of the induced pluripotent stem cell lines; CC1-1 to CC1-25: Each induced pluripotent stem cell line; SNV: Single-nucleotide variant.

Table 8 Genotypes of single-nucleotide variants among the matched adjacent non-cancerous tissue, the starting cancer tissue, and the cancer tissue-derived induced pluripotent stem cell lines

SNV No.	Genotypes of SNVs											
	NCC1	CC1	CC1-1	CC1-2	CC1-7	CC1-8	CC1-9	CC1-11	CC1-12	CC1-17	CC1-18	CC1-25
1	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/T	C/C
2	A/A	A/A	A/A	A/A	A/A	A/A	A/G	A/A	A/A	A/A	A/A	A/A
3	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/C
4	C/C	C/C	C/C	C/C	C/C	C/T	C/C	C/C	C/C	C/C	C/C	C/C
5	C/C	C/C	C/T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C
6	T/T	T/T	T/T	T/C	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T
7	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/T	G/G	G/G	G/G	G/G
8	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/C	G/G	G/G	G/G
9	C/C	C/C	C/C	C/C	C/C	C/T	C/C	C/C	C/C	C/C	C/C	C/C
10	C/C	C/C	C/C	C/T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C
11	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/A
12	A/A	A/G	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A
13	G/G	G/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G

NCC1: The matched adjacent non-cancerous tissue; CC1: The starting cancer tissue of the induced pluripotent stem cell lines; CC1-1 to CC1-25: Each induced pluripotent stem cell line; SNV: Single-nucleotide variant.

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COMMENTS

Background

Starting cells for induced pluripotent stem cell (iPSC) generation should be appropriately adopted to generate normal or aberrant iPSC lines for use in regenerative medicine or cancer research/therapy. Human iPSC lines for regenerative medicine would be ideally generated from normal neonatal tissues, as they are typically free of postnatal aberrant mutations and epigenetic changes. For cancer research/therapy, it is of great interest to generate iPSCs that originate from heterogeneous cancer tissues.

Research frontiers

Microarray experiments have profiled the gene expression of human iPSC lines clonally generated from a heterogeneous mixture of primary cells derived from gastric tissue or colon cancer tissue. The gene expression profiles of such iPSC lines demonstrate differences derived from their distinct starting tissues and

similarity and heterogeneity derived from their common starting heterogeneous tissue.

Innovations and breakthroughs

This is the first study to analyze human iPSC lines clonally generated from a heterogeneous mixture of primary cells derived from cancer tissues using next-generation sequencing. Eight of the ten iPSC lines had single-nucleotide variants with *de novo* or pre-existing mutations originating from a minor population within the cancer tissues. Meanwhile, all other genotypes of the iPSC lines were not mutated as in the original cancer tissues. Two of the ten iPSC lines did not possess any confirmed mutated genotypes despite having been derived from cancer tissue. These results suggest that the majority of iPSC lines originated from starting cells other than major cancer cells. Thus, the genotypes of iPSC lines can be used to trace the genotypes of the starting single cells.

Applications

It is conceivable that cancer tissues are made up of not only pre-cancer (stem) cells and pre-metastatic cancer cells but also stroma cells (such as mesenchymal stem cells, cancer-associated fibroblasts and tumor endothelial cells) and immune cells (such as tumor-associated macrophages, dendritic cells and tumor-infiltrating T cells). These other cell types might serve as targets for drug discovery and immune-cell therapy against cancer. Therefore, such a cell-derived iPSC line might be useful for immune-cell therapies such as cancer vaccines, dendritic cells and tumor antigen-specific cytotoxic T cells, in addition to the development of models of carcinogenesis and drug discovery tools.

Terminology

Most single-nucleotide variants are heteroallelic genotypes that are validated with allelic depth and genotype quality and manually curated using the Integrative Genomics Viewer. Deeper allelic depth of next-generation sequencing further resolves genotype variations among the starting single cells present within heterogeneous cancer tissues. In this way, the genotypes of the iPSC lines may be used to trace the genomic identity of their starting single cells derived from a heterogeneous cancer tissue.

Peer-review

The manuscript is well written and easy to follow.

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Skeletal muscle generated from induced pluripotent stem cells - induction and application

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Abstract

Human induced pluripotent stem cells (hiPS cells or hiPSCs) can be derived from cells of patients with severe muscle disease. If skeletal muscle induced from patient-iPSCs shows disease-specific phenotypes, it can be useful for studying the disease pathogenesis and for drug development. On the other hand, human iPSCs from healthy donors or hereditary muscle disease-iPSCs whose genomes are edited to express normal protein are expected to be a cell source for cell therapy. Several protocols for the derivation of skeletal muscle from human iPSCs have been reported to allow the development of efficient treatments for devastating muscle diseases. In 2017, the focus of research is shifting to another stage: (1) the establishment of mature myofibers that are suitable for study of the pathogenesis of muscle disease; (2) setting up a high-throughput drug screening system; and (3) the preparation of highly regenerative, non-oncogenic cells in large quantities for cell transplantation, *etc.*

Key words: Human induced pluripotent stem cells; Skeletal muscle; Transplantation; Disease; Modeling; Muscle progenitors; Muscular dystrophy; MYOD

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Core tip: Skeletal muscle cells induced from patient induced pluripotent stem cells (iPSCs) are useful for the study of pathogenesis and drug development. The derivation of mature myofibers is required for disease modeling. On the other hand, human iPSCs from healthy donors are likely to be a cell source for cell therapy. For safe cell transplantation, non-oncogenic cells are needed.

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INTRODUCTION

In 2006, Takahashi *et al.*^[1] reported that they successfully reprogrammed skin fibroblasts into pluripotent stem cells, which are undistinguishable from embryonic stem (ES) cells, using *oct4*, *sox2*, *klf4* and *c-myc*. They called this new pluripotent stem cell type "induced pluripotent stem cells (iPSCs)". Human induced pluripotent stem cells (hiPSCs) are rejuvenated, proliferate *in vitro* keeping their pluripotency, and differentiate into multipotent cell lineages. As a result, the induced pluripotent stem (iPS) technology was expected to advance the study of pathogenesis, drug screening, and regenerative medicine. However, in the field of skeletal muscle disease, the use of iPSCs has been relatively limited due to the difficulty of inducing skeletal muscle cells from human iPSCs in large quantities with sufficient purity. In addition, skeletal muscle derived from human iPSCs generally show embryonic phenotypes. In this review, we try to summarize the recent progress and remaining problems to be solved in inducing muscle cells from human iPSCs and their application.

MUSCLE SATELLITE CELLS/MYOBLAST-BASED CELL THERAPY

Muscle satellite cells are skeletal muscle-specific stem cells that reside between the muscle basement membrane and the plasma membrane of myofibers in a G₀ state in adult muscle. When muscle is damaged, satellite cells are activated, proliferate (myoblasts), and fuse with injured myofibers to repair muscle tissue. In Duchenne muscular dystrophy (DMD), however, muscle satellite cells are exhausted by repeated cycles of muscle degeneration and regeneration^[2,3]. As a result, myofibers are replaced by fibrotic tissue and adipocytes. In 1989, Partridge *et al.*^[4] demonstrated that direct injection of normal myoblasts into mdx muscle converted dystrophin-negative myofibers to dystrophin-positive ones. Based on this finding, clinical trials of myoblast transplantation therapy (MTT) were performed. However, MTT for DMD conducted between 1991 and 1997 was not successful^[5-7]. Experiments using mouse models suggested the rapid and massive death of a substantial fraction of injected myoblasts after transplantation^[8]. It was also demonstrated that satellite cells lose their regenerative ability during expansion in culture^[9,10]. Because it is not possible to prepare fresh myoblasts in large quantities from limited donor muscle tissues, MTT is now applied to myopathies

that affect specific muscles, such as those in oculopharyngeal muscular dystrophy^[11].

IPSC-BASED CELL THERAPY

Although it has long been difficult to induce skeletal muscle from human ES/iPSCs, several groups have recently reported successful derivation of skeletal muscle^[12]. Many researchers expect that iPS technology will overcome the limitations of MTT because iPSCs are expected to provide a large quantity of muscle progenitor/precursor cells without invasive procedures. It is also expected that more proliferative and regenerative stem/progenitor cells can be induced from hiPSCs than from postnatal myoblasts.

INDUCTION OF MYOGENIC PROGENITORS AND PRECURSOR CELLS FROM HUMAN IPSCS

The protocols for the derivation of skeletal muscle from human ES/iPSCs can be roughly divided into two categories: (1) direct reprogramming with muscle-specific transcription factors, such as PAX3, PAX7; and MYOD; and (2) the step-wise induction of skeletal muscle using small molecules and cytokines to inhibit or activate relevant signaling pathways in myogenesis (Figure 1).

Forced expression of MYOD or PAX7

More than 25 years ago, Weintraub *et al.*^[13] found that MyoD can convert non-myogenic cells to skeletal muscle cells^[13]. Rao *et al.*^[14] lentivirally transduced human ES cells with a doxycycline (DOX)-inducible MyoD. Within 10 d after addition of DOX to the culture, multinucleated myotubes were formed. The induction efficiency was over 90%. Tanaka *et al.*^[15] used a Piggy Bac transposon vector to overexpress MYOD and showed robust induction of skeletal muscle from Miyoshi myopathy-iPSCs. Akiyama *et al.*^[16] reported that transient ectopic expression of a catalytic domain of histone demethylase JMJD3, which reduces H3K27me, together with synthetic MyoD mRNAs, further accelerates the differentiation of human pluripotent stem cells into myogenic cells. Thus, MyoD-mediated muscle induction is fast and efficient. A limitation of the method would be that a high level or long expression of MyoD protein induces cell cycle arrest. In addition, MyoD cannot induce PAX3+PAX7+ muscle progenitors. For *in vitro* disease modeling, the properties of myotubes induced by the forced expression of MyoD remain to be compared with myotubes induced by Stepwise methods *via* the paraxial mesoderm and somite stage.

Pax3 and Pax7 regulate skeletal muscle formation during development, but play distinct roles in the post-natal period (reviewed in Ref.^[17]). Forced expression of PAX7 in embryoid bodies successfully induces transplantable myogenic cells from human ES cells^[18]. In contrast to MYOD, however, PAX7 alone does not

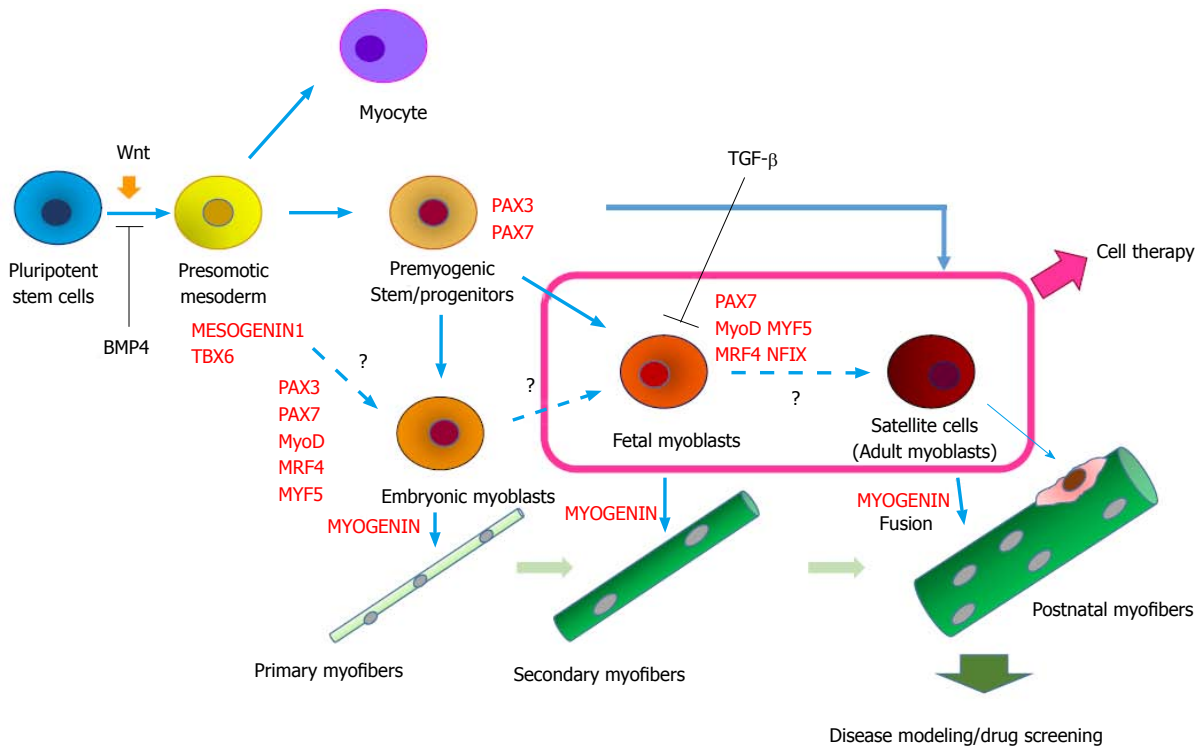


Figure 1 Step-wise induction of skeletal muscle from human embryonic stem/induced pluripotent stem cells and their application. In many protocols, pluripotent stem cells are first induced to differentiate into paraxial mesoderm using a GSK3 inhibitor (activation of Wnt signal) and a BMP4 inhibitor, and they then differentiate into premyogenic progenitors in serum-free DMEM/F12-ITS (or KSR) medium supplemented with growth factors such as FGF-2, IGF-1, or HGF. After differentiation into muscle progenitors, the cells are induced to precursor cells (myoblasts) and then differentiate into multinucleated myotubes (*in vitro*) and myofibers (*in vivo*). The transition from embryonic to fetal myoblasts and finally into adult myoblasts is thought to occur sequentially in a dish, but the mechanisms and modes are largely unknown. FGF: Fibroblast growth factor; IGF: Insulin-like growth factor; HGF: Hepatocyte growth factor.

convert adult fibroblasts to skeletal muscle. Therefore, the embryoid body would be the best stage in which to transduce myogenic cells with a PAX7-expression vector. Although random integration into the genome of over-expression vectors is not suitable for cell therapy, MyoD-induced skeletal muscle is now widely used for *in vitro* muscle disease modeling and drug screening.

Sphere-based culture method

Hosoyama *et al.*^[19] reported a sphere-based culture method for the derivation of myogenic progenitor cells from human ES/iPSCs (EZ sphere method). Human ES/iPSCs are cultured as spheres in serum-free medium for neurospheres supplemented with relatively high concentrations of fibroblast growth factor-2 and epidermal growth factor. After a six-week free-floating culture, cells plated onto Matrigel™-coated dishes start to form multinucleated myotubes and finally start to twitch. EZ-sphere cells contain both myogenic cells and neural cells, requiring the purification of myogenic cells for further application. In addition, whether the EZ-sphere method can induce transplantable myogenic cells or not remains to be shown.

Embryoid body-based induction

Awaya *et al.*^[20] reported a method for the selective expansion of mesenchymal cells from cell aggregation called embryonic bodies (EBs). The resulting cells

express CD56 (N-CAM) and the mesenchymal markers CD73, CD105, CD166, and CD29. The cells are transplanted into the muscle of immune-deficient mice and regenerate myofibers, as well as replenish the satellite cells. This method and the EZ sphere method require a lengthy culture and are not highly efficient.

Induction of skeletal muscle via activation of Wnt signaling

Many successful methods to induce skeletal muscle progenitors use a GSK-3 β inhibitor (which activates Wnt signaling) in the first phase of culture^[21-25]. Chal *et al.*^[24,25] monitored the induction process using reporter iPSC lines and comprehensive gene expression analysis, and established a stepwise induction of skeletal muscle. Paraxial mesoderm specification was achieved using a GSK3 inhibitor (CHIR-99021) together with BMP4 inhibition (LDN-193189) because BMP4 inhibition prevents the cells from differentiating into lateral mesoderm. The method induces myogenin(+) myogenic cells with 25%-30% efficiency^[24,25]. The induced myotubes express titin, a fast perinatal myosin heavy chain, have a sarcomere structure, and spontaneously contract^[24,25].

Heterogeneity of differentiation potential of human iPS clones

Human iPSCs are heterogeneous in myogenic differen-

tiation potential. Some iPS clones efficiently differentiate into the skeletal muscle lineage, while others do not. The heterogeneity is found even among iPS clones derived from the same donor using the same method. Although the molecular basis is largely unknown, one possibility is that some clones are incompletely reprogrammed and cannot respond to differentiation signals properly. If the induction protocol is appropriate, completely reprogrammed iPS clones are expected to efficiently differentiate into the skeletal muscle lineage. Recently, using integrative analysis of reprogramming in a human isogenic system, Shutova *et al.*^[26] identified criteria to select the best iPS line.

CHARACTERIZATION OF INDUCED MUSCLE CELLS

In humans, the myogenesis process can be divided into 3 developmental stages: primary myogenesis (6-8 wk of development), secondary myogenesis (8-18 wk of development) and adult-type myogenesis (muscle growth in later myogenesis and regeneration). In primary myogenesis, embryonic myoblasts form primary myofibers. In secondary myogenesis, fetal myoblasts form secondary myofibers. Postnatally, satellite cells fuse with growing myofibers or fuse with injured myofibers^[27,28]. During regeneration, a fraction of satellite cells return to their niche (self renewal) and maintain quiescence until the next turn. Importantly, the developmental stage of the myogenic progenitors largely determines the types of myofibers they form.

Morphology and gene expression of hiPSC-derived muscle

Human embryonic myoblasts show a limited proliferation capacity and are more prone to differentiation than fetal myoblasts. Isolated embryonic myoblasts form thinner myofibers with fewer nuclei than fetal myoblasts *in vitro*^[27-29]. Because embryonic and fetal myoblasts express quite different gene sets in mice^[28,30], gene expression analysis would be informative to determine the properties of the myogenic cells induced from human ES/iPSCs. For example, research in mice has revealed that embryonic myoblasts express PAX3, Paraxis, Meox1, Eya2, and Cadherin11, while fetal myofibers express NFIX, a key transcriptional regulator in fetal myoblasts^[31], MCK, PKC theta, HeyL, and integrin $\alpha 7$ ^[27,28,30]. These genes are good markers to determine the developmental stages of hiPSC-derived myogenic cells.

Cell surface markers

Cell surface markers to prospectively enrich myogenic progenitor cells with a highly myogenic and long-term expansion potential are under investigation. Barberi *et al.*^[32] reported the sorting of CD73(+) cells enriched in adult mesenchymal stem cell-like cells, and after 4-wk culture in ITS medium, NCAM(+) cells were collected and successfully transplanted into immunodeficient mice.

Borchin *et al.*^[22] reported that the sorting of cMet(+) CXCR4(+) ACHR(+) cells enriched myogenic progenitors. After the screening of more than 300 antibodies, Uezumi *et al.*^[33] found novel surface markers on adult myoblasts (CD82 and CD318) and succeeded in the enrichment of myogenic cells induced from hiPSCs using CD82. The new marker CD82 ensures expansion and preservation of myogenic progenitors by suppressing excessive differentiation of adult myoblasts. Alexander *et al.*^[34] also reported that CD82 is a marker for prospectively isolating stem cells from human fetal and adult skeletal muscle and is possibly involved in the pathogenesis of muscular dystrophies. The function of CD318 in myogenesis and whether CD318 is helpful for purification of hiPSC-derived myogenic cells are now under investigation.

Response to TPA, BMP-4, TGF- β and Notch

In mice, embryonic, fetal, and adult myoblasts have been demonstrated to respond differently to extracellular signals such as TPA, BMP-4, and TGF- β ^[27,28,35]. It was also shown that an activated Notch pathway is necessary for TGF- β - or BMP-4-mediated inhibition of differentiation in fetal myoblasts^[27,28]. By contrast, embryonic myoblasts are insensitive to TGF- β and BMP-mediated inhibition of differentiation^[27,28]. TPA inhibits the differentiation of fetal myoblasts, but not that of embryonic myoblasts and satellite cells, possibly through the activation of PKC^[27,28,36]. The PDGF receptor was reported to be expressed in embryonic myoblasts and adult myoblasts, but not in fetal myoblasts in the chick, suggesting that PDGF is involved in regulation of the transition of myogenesis^[27,28,37]. Such different sensitivities to external stimuli not only explain the different timings of the differentiation of embryonic, fetal, and adult myoblasts during development but are also informative to make engrafted myoblasts participate efficiently in muscle repair.

CELL TRANSPLANTATION OF IPSC-DERIVED MUSCLE PROGENITOR CELLS

Allogeneic transplantation of immune-compatible donor cells vs genome-edited autologous cell transplantation

Although the extent to which patient-derived iPSCs and their derivatives evoke immune reactions when transplanted into the same patient is still unclear^[38,39], recent tools for genome editing, such as CRISPR/Cas9, help in the preparation of gene-corrected cells from patients for autologous cell transplantation. For DMD, gene correction by homologous recombination is ideal, but restoration of the reading frame by exon skipping at the genomic level or by inserting a small DNA fragment is another option to obtain autologous, functional cells^[40]. Recently, Young *et al.*^[41] demonstrated a large CRISPR/Cas9-mediated deletion of 725 kb of DMD (deletion of DMD exon 45-55), resulting in reframed and functional DMD iPSCs. Genome editing can also generate PAX7 or MYF5 reporter iPSC lines to monitor

muscle differentiation^[42,43] or disease-specific iPSCs carrying various gene mutations in the same genetic backgrounds. On the other hand, hiPS stocks are under construction for allogeneic transplantation of immune-compatible donor cells (<https://www.cira.kyoto-u.ac.jp/e/research/stock.html>). The use of iPS stock of a guaranteed quality is less time consuming and more economical.

Xenotransplantation

Thus far, a limited number of reports have described the efficient engraftment of human iPSCs-derived myogenic cells in animal models^[18,20,21,24]. Most studies have used immune-deficient, dystrophin-deficient *mdx* mice as recipients. Recently, NSG-*mdx*^{4Cv} mice have been developed for xenotransplantation. NSG mice were generated by mating NOD/Scid mice with IL2 receptor gamma chain-null mice. NSG mice were then crossed with *mdx*^{4Cv} mice^[44]. The Central Institute for Experimental Animals in Japan established NOG (NOD/Shi-*scid*/IL-2Rγ^{null})-*mdx* mice, which have a different mutation in the IL-2 receptor gamma gene, and are also expected to be good recipients of human iPSC-derived muscle progenitor cells (https://www.ciea.or.jp/about/reports/pdf/report/59_report.pdf). In many studies of xenogeneic transplantation, the hindlimb muscles of host mice are X-irradiated to kill endogenous satellite cells. A highly toxic venom, cardiotoxin, or BaCl₂ is also used to damage the TA muscle 24 or 48 h before cell transplantation. Both X-irradiation and cardiotoxin injection effectively increase the contribution of engrafted cells to muscle regeneration; however, the effect is not physiological and cannot be applied to human recipients.

Delivery

Because most muscular dystrophies affect muscles of the whole body, the final goal of cell therapy is to deliver myogenic progenitors *via* the circulation. However, satellite cells and myoblasts cannot be delivered *via* the circulation. Mesoangioblasts have been reported to be systemically delivered and ameliorate dystrophic phenotypes in murine and canine models^[45,46]. Therefore, the induction of mesoangioblasts from human ES/iPSCs is an attractive strategy to target the whole musculature. Tedesco *et al.*^[47] reported induction of mesoangioblast-like myogenic cells from iPSCs. Because the iPSC-derived mesoangioblast-like cells did not spontaneously differentiate into skeletal muscle, the authors overexpressed MyoD-estrogen receptor fusion protein in them and induced myogenic differentiation by tamoxifen administration after intramuscular transplantation.

EVALUATION METHOD FOR PROOF-OF-CONCEPT IN XENOTRANSPLANTATION

Histological and immunohistochemical analysis

Reduced necrotic fibers (H and E staining), fibrosis

(Masson's trichrome), and adipogenesis (oil red O), increased fiber diameter and muscle mass, and reduced inflammation are all indicative of the therapeutic effects of cell therapy. The percentage of central nuclei is not suitable for evaluation because, once myofibers regenerate, nuclei stay in the central position for a long time. Myofibers formed by transplanted cells are immunohistochemically detected using antibodies against human proteins, such as human laminA/C (nuclear membrane) or human spectrin (sarcolemma). The widely used human spectrin antibody (clone RBC2/3D5) reacts with mouse utrophin^[48], and dystrophin recognizes revertant fibers. In fact, we experienced high levels of dystrophin expression in NSG-*mdx*^{4Cv} mice (0.84% in the TA muscle of 6-mo-old males) (data not shown). Rozkalne *et al.*^[48] advised against relying on the detection of a single protein, but performing multiple human-specific labels and detecting dystrophin and dystrophin-associated proteins at the sarcolemma instead.

Muscle function

The improvement of muscle function is the most reliable proof-of-concept for cell therapy of muscular dystrophy. The measurement of the tetanic and specific force of an isolated single myofiber or muscle tissues *in vitro* is one of the widely used evaluation methods, but it is technically difficult. To obtain reproducible data, a system was developed in which the torque of the ankle of mice (planter flexion) is measured after the injection of myogenic stem/progenitor cells into gastrocnemius muscles. The measurement can be performed under anesthesia at different time points (http://www.brck.co.jp/application/files/3614/1523/5703/BRCsogo20-11_P145.pdf).

IN VIVO SURVIVAL AND DIFFERENTIATION OF TRANSPLANTED CELLS

The efficiency of the transplantation of muscle stem/progenitor cells depends both on the intrinsic properties of the transplanted cells and on the microenvironment in the diseased muscle. Sakai *et al.*^[49] reported that mouse satellite cells showed many more dystrophin-positive fibers than mouse fetal muscle progenitors after intramuscular transplantation into dystrophin-deficient *mdx* mice. By contrast, Tierney *et al.*^[50] demonstrated that fetal muscle stem cells expand and contribute to muscle repair more efficiently than satellite cells after transplantation. Although the reasons for the discrepancy in the results are unclear, the studies suggest that the efficiency of transplantation depends largely on the intrinsic properties of the cells. Therefore, it is important to determine the signals that differently regulate the survival, proliferation, and differentiation of muscle stem/progenitor cells derived from hiPSCs. In addition, the microenvironment of diseased muscle might inhibit the survival and differentiation of engra-

fted cells. For example, fibrosis, an impaired blood supply, an inflammatory environment, and an activated immune response all inhibit the ability of engrafted cells to survive, proliferate, and differentiate to fuse host myofibers. The reconstitution of a regeneration-friendly microenvironment using a scaffold filled with regeneration-supportive ECM and cytokines, and the suppression of inflammatory responses would be effective.

TUMOR FORMATION BY IPS CELL-DERIVED MYOGENIC CELLS

Tumor-like growth in the host muscle after the transplantation of hiPS-derived muscle progenitor cells is occasionally observed. However, few publications have examined this problem extensively. In our opinion, the causes of tumorigenesis can be divided into at least three categories. The first cause is residual pluripotent stem cells in the transplanted population, which form teratomas. Teratoma is rare, and the elimination of undifferentiated pluripotent cells using FACS and human ES/iPSC markers such as SSEA4 or TRA-1-60 or by a recombinant lectin-toxin fusion protein would be effective^[51]. The second cause is genetic abnormalities of human iPSCs ranging from gross karyotypic abnormalities to sub-chromosomal abnormalities (gene duplication, deletions, point mutation, *de novo* copy number variations (CNVs)). Mutations are reported to occur during the derivation and culture of human ES/iPSCs and are supposed to be responsible for tumor formation after the transplantation of hiPS-derived progenitor cells^[52,53]. Re-activation of transgenic oncogenes like *c-Myc* or *KLF4* used for reprogramming is often related to the overgrowth of transplanted cells. These genetic abnormalities should be carefully examined before clinical application. The third cause is immature progenitors that fail to differentiate into mature cells for unknown reasons and continue to proliferate in transplanted tissues. In fact, we occasionally observed that hiPSC-derived myogenic cells overgrew in the muscle of immune-compromised mice. A similar phenomenon was observed in the transplantation of neurogenic cells. Interestingly, Ogura *et al.*^[54] reported that a Notch inhibitor promoted the differentiation of immature, actively proliferating progenitors, resulting in reduced tumor-like growth and engraftment of mature neurons in animal models of Parkinson's disease. Similar results have been reported in a mouse model of spinal cord injury^[55]. Whether such differentiation-resistant neuronal progenitor cells carry specific genetic abnormalities is not clear. Detailed characterization of cells that proliferate without terminal differentiation in transplanted muscle and investigation of the signaling pathways controlling self-renewal and differentiation of progenitors would be needed.

DISEASE MODELING IN VITRO USING PATIENT-DERIVED IPSCS

Successful examples of disease modeling

iPSCs derived from patients are useful for the elucidation of molecular pathogenesis and drug discovery. Various muscle disease-specific iPSCs have already been generated and deposited in a cell bank (e.g., <http://cell.brc.riken.jp/en/>; <https://catalog.coriell.org/>). The CRISPR/Cas9 technique further widened the possibility of examining the molecular pathology of ultra-rare diseases. For Duchenne muscular dystrophy (DMD), several groups have already reported that DMD-iPSCs-derived muscle cells show disease-specific phenotypes *in vitro*; Choi *et al.*^[56] reported the aberrant expression of inflammation or immune-response genes and reduced fusion competence of DMD-iPS-derived myogenic cells. Shoji *et al.*^[57] reported a pronounced calcium ion influx only in DMD myotubes, which were rescued by morpholino-mediated exon-skipping to skip a premature stop codon. Chal *et al.*^[24] reported that fibers derived from the ES cells of mdx mice exhibited an abnormal branched phenotype resembling that described *in vivo*. For other muscular dystrophies, Tanaka *et al.*^[15] demonstrated defective membrane repair in hiPSC-derived myotubes from a Miyoshi myopathy patient and phenotypic rescue by the expression of full-length DYSFERLIN. Snider *et al.*^[58] reported that hiPSCs express full-length DUX4, and the differentiation of control iPSCs to embryoid bodies suppresses the expression of full-length DUX4, whereas the expression of full-length DUX4 persists in differentiated iPSCs derived from patients with FSHD (facio-scapulo-humeral muscular dystrophy). Iovino *et al.*^[59] have created a novel cellular model of human muscle insulin resistance by differentiating iPSCs from individuals with mutations in the insulin receptor into functional myotubes and characterizing their response to insulin compared with controls. These successful *in vitro* disease models using patient-iPSCs are encouraging and useful for screening new drugs.

Neuromuscular junction

Maturation of skeletal muscle derived from human iPSCs *in vitro* is generally limited, partly because myofibers mature under innervation. However, neuromuscular junction (NMJ) formation *in vitro* is still challenging^[60]. Morimoto *et al.*^[61] reported three-dimensional (3D) free-standing skeletal muscle fibers co-cultured with motor neurons. Yoshida *et al.*^[62] generated an NMJ-like structure using motor neurons derived from SMA patient-specific iPSCs and myotubes formed by C2C12 cells. Importantly, the clustering of acetylcholine receptors (AChR) is severely impaired. The authors further showed that valproic acid or antisense oligonucleotide-targeting splice-silencing motifs in intron 7 of *SMN2* ameliorated the AChR clustering defects, by increasing the level of

SMN2 transcripts^[62].

Mechanical stress

Mechanical stress is needed for the maturation of hiPSC-derived muscle. A decellularized ECM scaffold filled with hiPSC-derived muscle progenitor cells might help us to obtain functional skeletal muscle tissue under physiological mechanical stress.

Induction of diverse myofibers in the body

Our musculature is composed of many types of muscle in the body: Cranial muscle, trunk muscle, and limb muscle. They have different developmental origins and programs. Each muscle is composed of slow or fast myofibers expressing different types of myosin heavy chain genes^[63]. To faithfully mirror the physiology and pathology *in vivo*, such differences should be considered, although an induction method for diverse types of myofibers is at present challenging.

CONCLUSION

To maximally utilize the benefits of iPS technology for the cell therapy of devastating muscle disorders, a standardized protocol to constantly and efficiently induce skeletal muscle stem/progenitor cells from hiPSCs in a short time at low cost is desirable. Reduction of the risk of tumorigenesis and systemic delivery of therapeutic cells to the wider musculature are also required, and they are still highly challenging. For the modeling of disease, maturation of myotubes into adult-type myofibers *in vitro*, including the reconstitution of the neuromuscular junction, would be helpful.

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ORIGINAL ARTICLE

Basic Study

- 98 Modifying oxygen tension affects bone marrow stromal cell osteogenesis for regenerative medicine

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Basic Study

Modifying oxygen tension affects bone marrow stromal cell osteogenesis for regenerative medicine

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Author contributions: Inagaki Y and Akahane M designed the study; Inagaki Y, Akahane M, Shimizu T, Inoue K, Egawa T and Kira T performed the study; Inagaki Y and Akahane M drafted the manuscript; Ogawa M, Kawate K and Tanaka Y critically reviewed the draft; all authors read and approved the final manuscript.

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Abstract**AIM**

To establish a hypoxic environment for promoting osteogenesis in rat marrow stromal cells (MSCs) using osteogenic matrix cell sheets (OMCSs).

METHODS

Rat MSCs were cultured in osteogenic media under one of four varying oxygen conditions: Normoxia (21% O₂) for 14 d (NN), normoxia for 7 d followed by hypoxia (5% O₂) for 7 d (NH), hypoxia for 7 d followed by normoxia for 7 d (HN), or hypoxia for 14 d (HH). Osteogenesis was evaluated by observing changes in cell morphology and calcium deposition, and by measuring osteocalcin secretion (ELISA) and calcium content. *In vivo* syngeneic transplantation using OMCSs and β -tricalcium phosphate discs, preconditioned under NN or HN conditions, was also evaluated by histology, calcium content measurements,

and real-time quantitative PCR.

RESULTS

In the NN and HN groups, differentiated, cuboidal-shaped cells were readily observed, along with calcium deposits. In the HN group, the levels of secreted osteocalcin increased rapidly from day 10 as compared with the other groups, and plateaued at day 12 ($P < 0.05$). At day 14, the HN group showed the highest amount of calcium deposition. *In vivo*, the HN group showed histologically prominent new bone formation, increased calcium deposition, and higher collagen type I messenger RNA expression as compared with the NN group.

CONCLUSION

The results of this study indicate that modifying oxygen tension is an effective method to enhance the osteogenic ability of MSCs used for OMCSs.

Key words: Hypoxia; Osteogenesis; Tissue engineering; Marrow stromal cells; Regenerative medicine

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Core tip: Bone tissue engineering using marrow stromal cells (MSCs) is a promising method in regenerative medicine. Here, we have reported a scaffold-free transplantation technique using hypoxic-preconditioned osteogenic matrix cell sheets (OMCSs) derived from MSCs. We show that modifying the oxygen tension before implantation of OMCS composites led to an increased osteogenic capacity of rat bone MSCs.

Inagaki Y, Akahane M, Shimizu T, Inoue K, Egawa T, Kira T, Ogawa M, Kawate K, Tanaka Y. Modifying oxygen tension affects bone marrow stromal cell osteogenesis for regenerative medicine. *World J Stem Cells* 2017; 9(7): 98-106 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v9/i7/98.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v9.i7.98>

INTRODUCTION

Bone defects resulting from severe fracture, infection, or tumor resection are serious problems that impair patient quality of life. Bone tissue engineering is a novel way to produce sufficient bone to repair bony defects. Various methods^[1-5] have examined the use of marrow stromal cells (MSCs) as a cell source for bone tissue engineering, and several reports have incorporated natural or synthetic scaffolds to maintain MSCs^[6-8]. However, each method has its limitations, including possible immunological responses against natural materials, and reduced bioactivity or biocompatibility with synthetic materials^[9]. So, too, have several recombinant proteins, such as bone morphogenetic protein-2, been vigorously tested in conjunction with these scaffolding materials because of

their capacity to enhance osteogenesis. However, the use of recombinant proteins is limited, as it is difficult to control their dosage, with multiple applications or genetic manipulation, such as gene transfer, often required to accommodate their short half-life *in vivo*. Thus, more effective and simpler methods are needed to enhance osteogenesis for bone tissue reconstruction.

Oxygen tension affects the proliferation and differentiation of MSCs^[10,11], and cell culture conditions are generally set to approximately 21% O₂ and 5% CO₂ at 37 °C. However, several studies have reported that, in the bone marrow, physiological oxygen concentrations range from 6.6% to 8.6% (54.9 mmHg to 71.4 mmHg), as measured using a polarographic needle electrode^[12,13]. In a study focusing on the effect of hypoxia on MSC osteogenesis, Lennon *et al.*^[10] reported that 5% O₂ in primary and subcultures of rat MSCs could enhance osteogenesis. Conversely, D'Ippolito *et al.*^[11] have shown that 3% O₂ inhibits osteogenic differentiation of human MSCs. Thus, the ideal concentration, duration, and timing of hypoxic treatment to enhance osteogenesis remain unclear.

We previously reported several methods for musculoskeletal reconstruction using cell transplantation techniques using composites of artificial bone combined with MSCs, and the use of a scaffold-free cell sheets, referred to as osteogenic matrix cell sheets (OMCSs). As hypoxic treatment may enhance osteogenesis for such composites and cell sheets, in the present study, we evaluated whether hypoxia could enhance MSCs and the osteogenesis of OMCSs created from MSCs.

MATERIALS AND METHODS

Ethics statement

All experimental protocols using animals were approved by the Animal Experimental Review Board of the Nara Medical University before experimentation. Animals were housed in a temperature-controlled environment at approximately 21 °C under a 12-h light/12-h dark cycle with free access to food and water.

Bone marrow cell preparation

Seven-week-old male Fisher-344 rats (Japan SLC, Shizuoka, Japan) were purchased for bone marrow cell preparation. Bone marrow cells were obtained from the femoral shafts of rats as previously described^[1]. Briefly, both ends of the femurs were cut from the epiphysis, and the marrow was flushed with 10 mL of basal medium expelled from a syringe through a 21-gauge needle. Basal medium consisted of Eagle's Minimal Essential Medium (Nacalai Tesque, Kyoto, Japan), 15% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, United States) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin; Nacalai Tesque). The flushed cells were collected into two

T-75 flasks (Falcon, BD Biosciences, Franklin Lakes, NJ, United States) containing 15 mL of basal medium. Non-adherent cells were removed during media changes, which were performed three times per week. Cultures were maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C.

After reaching confluence, cells were released by trypsin/EDTA (Nacalai Tesque) and used for *in vitro* and *in vivo* experiments.

***In vitro* experiment**

Osteogenic cell culture and experimental design:

Cells were seeded at a density of 1×10^4 cells/cm² in 12-well cell culture plates (Falcon, BD Biosciences), and subcultured in osteogenic medium consisting of basal medium supplemented with 10 nmol/L dexamethasone (Sigma-Aldrich, St. Louis, MO, United States) and 0.28 mmol/L ascorbic acid phosphate (Wako Pure Chemical Industrials, Kyoto, Japan). These subcultured cells were exposed to one of four variable oxygen concentration conditions: Normoxia (21% O₂) for 14 d (NN), normoxia for the first 7 d followed by hypoxia (5% O₂) for the next 7 d (NH), hypoxia for the first 7 d followed by normoxia for the next 7 d (HN), and hypoxia for 14 d (HH). The entire experiment was repeated using cells from two different animals. The number of replicates within each assay.

Osteocalcin secretion measurement: Osteocalcin secretion was measured to evaluate the osteogenic potential of MSCs. Secreted osteocalcin is a reliable marker for predicting *in vivo* osteogenic potential for bone tissue engineering^[14]. Secreted osteocalcin levels were measured on days 7, 10, 12, and 14 using an enzyme-linked immunosorbent assay (ELISA) with anti-rat osteocalcin monoclonal antibody (DS Pharma Biomedical, Osaka, Japan). A media change performed 48 h before collection ($n = 5$ for each group).

Observations of cell morphology and calcium deposition: After 14 d of osteogenic culture, the media was replaced with phosphate-buffered saline (PBS; Gibco, Paisley, United Kingdom), and cell morphology and calcium deposition on the culture plates were observed using an inverted microscope (Eclipse Ti-S, Nikon, Tokyo, Japan). Images were taken using a digital camera (Digital Sight DS-Fi1, Nikon) ($n = 5$ for each group).

Measurement of calcium deposition: After observations of cell morphology and calcium deposition, total calcium was extracted from each well with 2.0 mL of 20% formic acid, and measured using a methylxlenol blue method (Calcium E-test Wako Kit, Wako Pure Chemical Industrials) ($n = 5$ for each group). Measurements were adjusted to the total amount of protein in each well, as determined using a spectrophotometric method with bovine serum albumin as a standard.

Alkaline phosphatase staining: For alkaline phosphatase (ALP) staining^[1], cells were cultured in osteogenic medium for 14 d in 6-well plates under variable oxygen conditions, rinsed twice with PBS, and then stained with naphthol-AS-MX phosphate sodium salt (Sigma-Aldrich) and fast red violet LB salt (Nacalai Tesque) at room temperature for 10 min. The stain was removed by rinsing with tap water, and air dried.

Cell proliferation assay: A colorimetric assay using tetrazolium salt was performed to assess the effect of hypoxic conditions on the proliferative capacity of MSCs. Cells were seeded at a density of 1×10^4 cells/cm² into the wells of a 96-well cell culture plate (Falcon, BD Biosciences) and subcultured in basal medium for 3 d under hypoxia or normoxia. Cell proliferation was then measured using a cell proliferation assay kit (Promega, Fitchburg, WI, United States), as per the manufacturer's recommendations ($n = 6$ for each group).

***In vivo* experiment**

Syngeneic OMCS transplantation: *In vivo* syngeneic transplantation experiments were performed using OMCSs prepared from second-passage bone marrow cells, as previously reported^[2,3]. OMCSs were exposed to one of two oxygen conditions: Normoxia (21% O₂) for 14 d (NN) or hypoxia (5% O₂) for the first 7 d followed by normoxia for the next 7 d (HN); these two conditions were chosen based on the results of the *in vitro* experiments. β -tricalcium phosphate (β -TCP) discs (Hoya, Tokyo, Japan; 75% porosity, 5-mm diameter, and 2-mm thickness) were wrapped with OMCSs and then implanted at subcutaneous sites on the backs of recipient rats.

In this experiment, OMCSs were wrapped around β -TCP discs so that the sheets could be easily identified and harvested for precise histological evaluation.

Histological evaluation: β -TCP discs with OMCSs were harvested after 4 wk. Samples were decalcified in K-CX solution (Falma, Tokyo, Japan), embedded in paraffin, cut at the middle of the specimen, and stained with hematoxylin and eosin (HE). Two authors (KI and TE), blinded to the grouping, evaluated the histological findings ($n = 5$ for each group).

Calcium content measurements: To quantify the amount of newly formed hydroxyapatite, harvested constructs were homogenized, and calcium content was measured as described earlier ($n = 5$ for each group).

Real-time quantitative PCR: Total RNA was extracted from harvested specimens using ISOGEN (Nippon Gene, Tokyo, Japan), and reverse transcribed into complementary DNA. Real-time quantitative PCR (Applied Biosystems Step One Plus Real Time PCR System, Thermo Fisher Scientific, MA, United

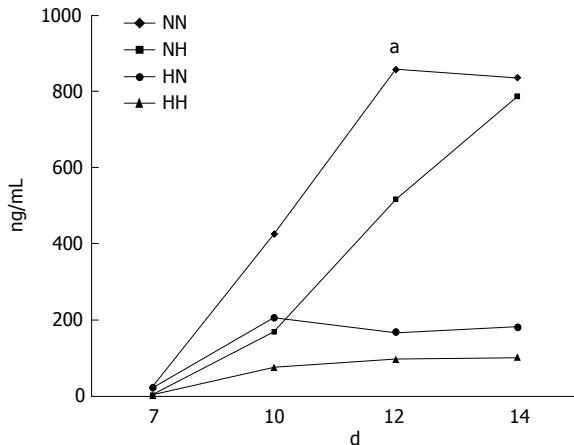


Figure 1 Osteocalcin secretion. In cells exposed to hypoxia (5% O₂) for 7 d followed by normoxia (21% O₂) for 7 d (HN), the level of osteocalcin increased rapidly from day 10, peaking at day 12, as compared with cells in the other groups. NN, normoxia for 14 d; NH, normoxia for 7 d followed by hypoxia for 7 d; and HH, hypoxia for 14 d. ^a $P < 0.05$.

States) was performed to measure the expression of collagen type I (Rn00801649 g1) and osteocalcin (Rn01455285 g1) ($n = 5$ for each group). Thermal cycling conditions were 20 s at 95 °C for activation (TaqMan Fast Universal PCR Master Mix, Thermo Fisher Scientific) followed by 40 cycles of 1 s at 95 °C for denaturation, and 20 s at 60 °C for annealing and extension. Expression levels were normalized to β -actin (Rn00667869 m1). All experiments were performed in duplicate.

Statistical analysis

Mann-Whitney *U*-test and one-way ANOVA with Bonferroni *post hoc* multiple comparisons were performed using SPSS Ver. 17.0 (IBM, Chicago, IL, United States). *P* values less than 0.05 were considered statistically significant for both tests.

RESULTS

In vitro experiment

Osteocalcin secretion measurement: At day 7, osteocalcin secretion levels were low in all four groups. In the HN group, levels started to increase rapidly at day 10, peaking and stabilizing from day 12 ($P < 0.05$). Levels also increased steadily for cells in the NN group, but remained low for cells in the NH and HH groups through day 14 (Figure 1).

Observations of cell morphology and calcium deposition: In the NN and HN groups, the differentiated cells appeared cuboidal, and cell nodules with calcium deposition were observed. In the NH and HH groups, however, few such nodules were seen. Instead, the cells in the NH and HH groups appeared spindle-shaped and undifferentiated, particularly in the HH group (Figure 2).

Calcium content measurements: At day 14, we compared the adjusted total calcium deposition in the culture wells among the four groups. The HN group showed the highest calcium deposition, followed by the NN group, as compared with HH and NH groups ($P < 0.05$) (Figure 3).

Alkaline phosphatase staining: The NH, HN, and HH groups showed broader positive staining areas as compared with the NN group. Among the three groups, the HN group showed the strongest and the broadest ALP staining. Staining intensity was weakest in the HH group (Figure 4).

Cell proliferation assay: Cell proliferation for cells grown under hypoxic conditions was significantly higher than that of cells grown under normoxic conditions ($P < 0.05$) (Figure 5).

In vivo experiment

Histological evaluation: HN constructs harvested from rats showed prominent new bone formation within the pores of the β -TCP discs. In contrast, there was less new bone formation observed in the NN constructs. This could be due to the reduced osteogenic ability of second-passage MSCs; although, the same cells were used for the HN group. The histological findings were consistent between the two blinded observers (KI and TE) (Figure 6).

Calcium contents measurement: HN constructs showed a significantly higher amount of calcium deposition as compared with the NN constructs (Figure 7).

Real-time quantitative PCR: We measured significantly higher expression of collagen type I mRNA in the HN constructs as compared with the NN constructs (Figure 8). Furthermore, there was a tendency for higher expression of osteocalcin mRNA, although this difference was not statistically significant.

DISCUSSION

Hypoxic environments affect all facets of cell function, including survival, proliferation, differentiation, migration, and endocrine and paracrine signaling. Hypoxia is mediated through the activity of transcription factors, most notably hypoxia inducible factor (HIF)^[15]. Because of the broad range of effects that hypoxia has on cell function, the effects of hypoxia on the osteogenic ability of MSCs has been controversial^[10,11]. In addition, differences in the experimental cell types and culture methods, and the timing of exposure to the hypoxic environment can lead to variation in findings among different studies. On the basis of our methods for regenerative medicine, we show that hypoxia (5% O₂) enhances cell proliferation when delivered for 7 d followed by normoxia for 7 d (HN group), with observed

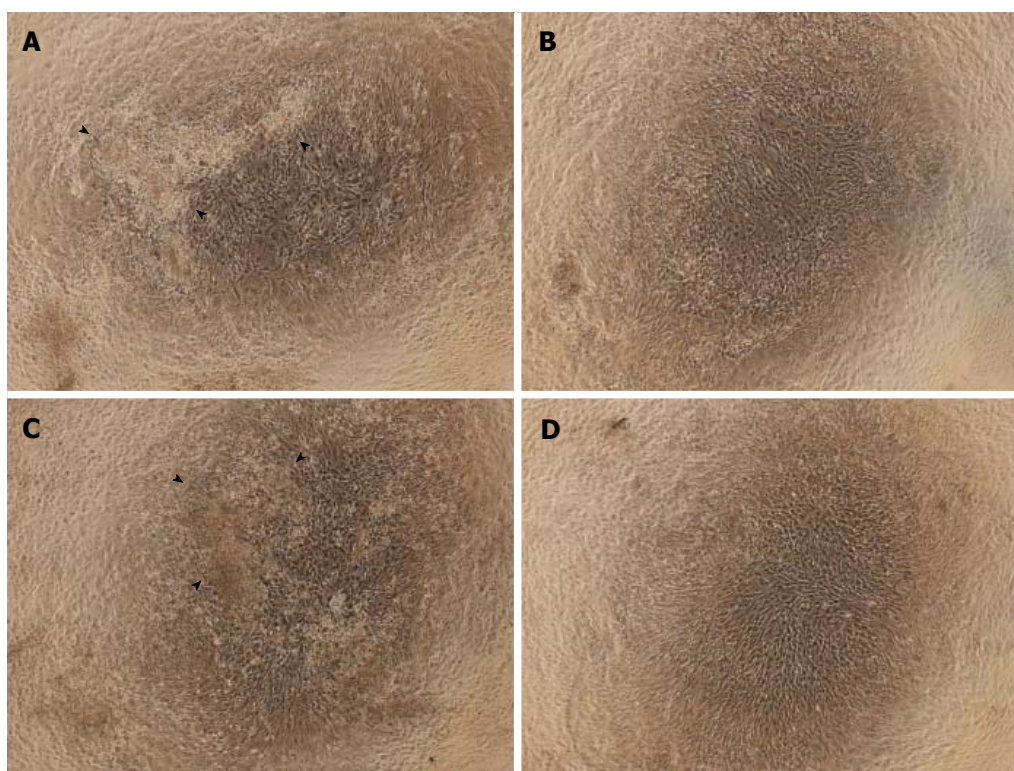


Figure 2 Observations of cell morphology and calcium deposition. Cells treated with normoxia (21% O₂) for 14 d (NN) (A) or hypoxia (5% O₂) for 7 d followed by normoxia for 7 d (HN) (C) were differentiated and cuboidal-shaped, and showed cell nodules with calcium deposits. These changes were not present in cells exposed to normoxia for 7 d followed by hypoxia for 7 d (NH) (B) or hypoxia for 14 d (HH) (D). Arrowheads indicate calcium deposition.

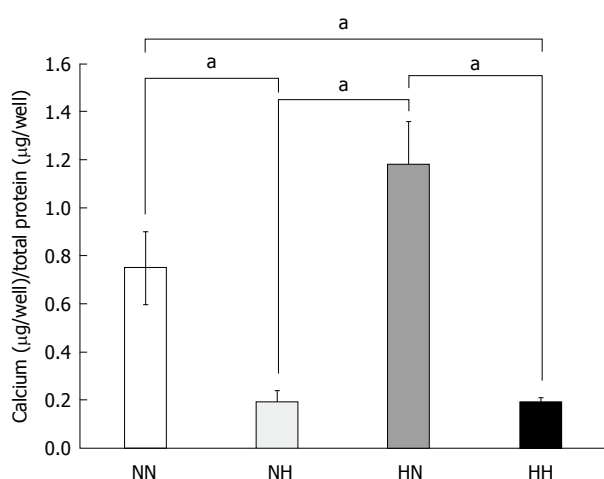


Figure 3 Calcium deposition. Cells were exposed to normoxia (21% O₂) for 14 d (NN), normoxia for 7 d followed by hypoxia (5% O₂) for 7 d (NH), hypoxia for 7 d followed by normoxia for 7 d (HN), or hypoxia for 14 d (HH). Calcium deposition was adjusted to total protein content. Cells in the HN group showed the highest amount of calcium deposition among the four groups. **P* < 0.05.

increases in osteocalcin secretion, calcium deposition, and ALP staining, suggestive of enhanced osteogenesis in MSCs. The results of our *in vivo* experiments also show that hypoxic pretreatment can drive higher rates of osteogenesis as compared with normal oxygen levels.

With regards to cell survival, others^[16] have shown that hypoxic preconditioning (0.5% O₂ for 24 h) both

in vitro and *in vivo* can increase the expression of pro-survival factors, Bcl-2 and Bcl-xL, and reduce cell death and caspase-3 activation in stem cells as compared with stem cells grown under normal oxygen conditions. Despite some contradictory results^[17,18], many other studies also support the positive effects of hypoxia (1.5%-8% O₂) on MSC proliferation^[10,11,19-22]. Here, we also showed enhanced MSC proliferation with hypoxic preconditioning, and suggest that the elevated osteogenic ability of the cells in the HN group may have arisen from the enhanced cell proliferation during the first 7 d of hypoxic culture.

D'Ippolito *et al.*^[11] previously reported an inhibitory effect of hypoxia on osteogenic differentiation of human marrow-isolated adult multilineage-inducible cells. However, they also reported that hypoxia shortened population doubling time, resulting in an increase in the rate of cell proliferation, which agrees with our results. However, in their study, hypoxia was employed throughout the culture period. In our study, we found that cells treated continuously under hypoxic conditions (HH group; 5% hypoxia for 14 d) showed lower proliferation, less osteogenic ability, and retained an undifferentiated morphology. Collectively, these data suggest that the appropriate timing of hypoxia is important for stimulating osteogenesis. To the best of our knowledge, our study is the first to seek the best temporal combination of hypoxia and normoxia during osteogenic culture.

Environmental preconditioning, such as through

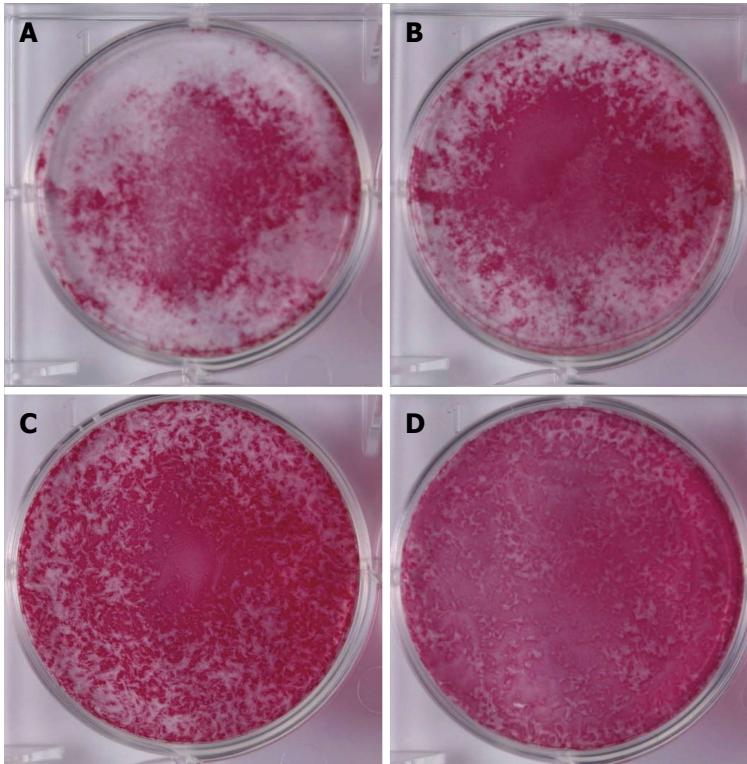


Figure 4 Alkaline phosphatase staining. Cells were exposed to normoxia (21% O₂) for 14 d (NN) (A), normoxia for 7 d followed by hypoxia (5% O₂) for 7 d (NH) (B), hypoxia for 7 d followed by normoxia for 7 d (HN) (C), or hypoxia for 14 d (HH) (D) and then stained with alkaline phosphatase staining. The HN group showed the strongest and the broadest alkaline phosphatase staining (C).

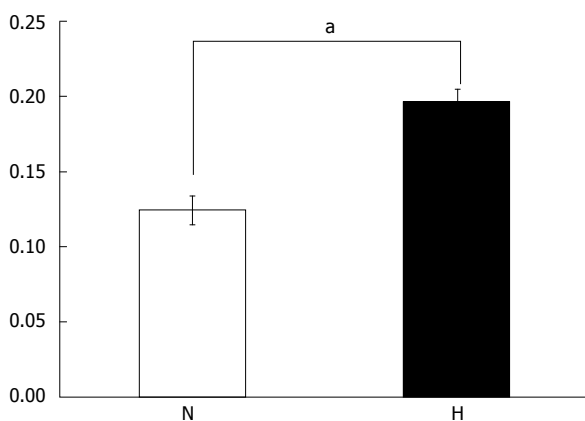


Figure 5 Cell proliferation assay. Cell proliferation under hypoxic conditions (H: 5% O₂) was significantly higher than that under normal oxygen conditions (N: 21% O₂). * $P < 0.05$.

the use of hypoxia or growth factor supplementation offers a potentially powerful approach to enhance the proliferative and differentiative ability of MSCs^[23]. Hagmann *et al.*^[24] added fibroblast growth factor-2 during human MSCs expansion, and found higher cell population growth indices and a downregulation of CD146, a marker of endothelial cells. Others have shown that decellularized extracellular matrix (dECM), deposited by stem cells, is another promising approach to create environmental preconditioning. He *et al.*^[25] construct a dECM by culturing porcine synovium-derived stem cells (SDSCs) on fibronectin-coated surfaces, and then lysed the cells using a nonionic surfactant. The authors then seeded SDSCs onto the dECM and found increased cell numbers and enhanced

chondrogenic capacity as compared with cells seeded on control substrates.

Numerous studies have reported the use of bio-engineered scaffolds for enhanced bone repair^[6-8]. However, considering the potential disadvantages of scaffolds, such as reduced biocompatibility^[9] or the possibility of causing an immunological response, we believe that scaffold- and recombinant protein-free techniques are better solutions. Recently, we reported the culture of MSCs as OMCSs, and demonstrated their osteogenic potential *in vitro* and bone formation *in vivo* in the absence of scaffolds^[2]. Furthermore, OMCSs can be used in non-union surgery and to accelerate bone-tendon healing in ligament reconstruction^[3]. Here, we showed that OMCSs preconditioned with hypoxic conditions offers a simple and inexpensive method to improve the osteogenic capacity of cells, and this may provide a better method for MSC-mediated bone tissue engineering.

Hypoxia as an environment preconditioning tool has been explored predominantly as a strategy to enhance the chondrogenic ability of MSCs^[23]. Human MSCs, preconditioned under hypoxic and normoxic environments, were encapsulated in alginate hydrogels and implanted subcutaneously onto the backs of nude mice. Whereas the hypoxia-preconditioned implants retained a chondral phenotype after implantation, the normoxia-preconditioned implants underwent calcification, vascular invasion, and subsequent endochondral ossification^[26]. Jukes *et al.*^[27] also utilized endochondral ossification to engineer bone tissue from mouse embryonic stem cells; however, the authors did not modulate the oxygen tension. We speculate that the

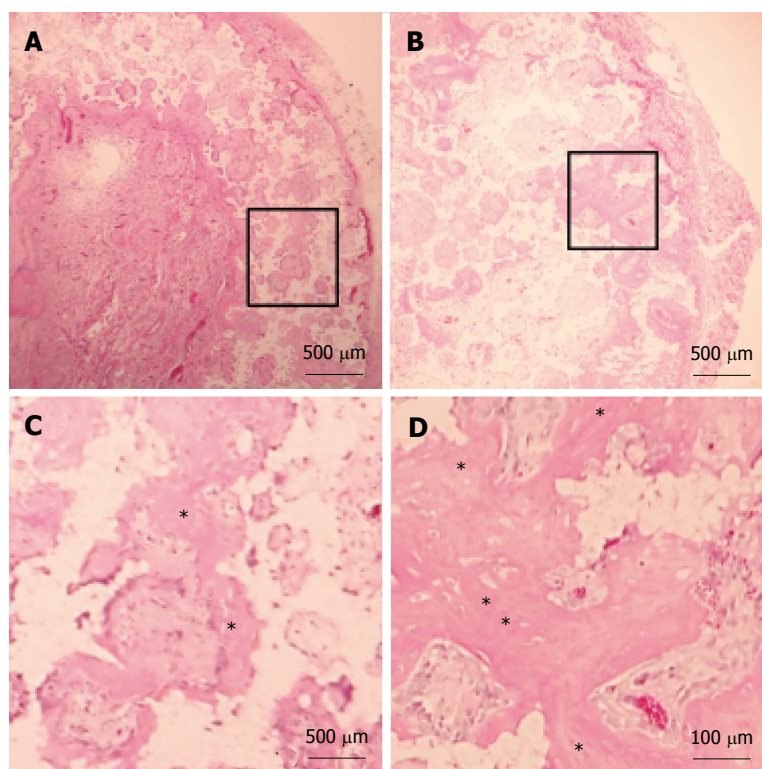


Figure 6 Histology of β -tricalcium phosphate discs wrapped with osteogenic matrix cell sheets. Cell sheets were exposed to hypoxia (5% O_2) for 7 d followed by normoxia (21% O_2) for 7 d (B) or normoxia for 14 d (A). Prominent newly formed bone (*) was observed in the HN group. (C) and (D) are higher magnifications of squared areas indicated in (A) and (B), respectively.

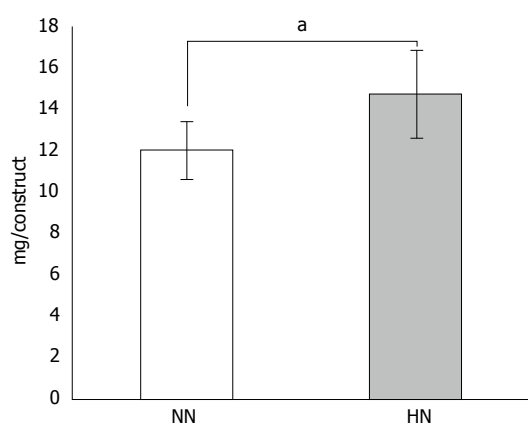


Figure 7 Calcium content in β -tricalcium phosphate discs wrapped with osteogenic matrix cell sheet. Cell sheets were exposed to hypoxia (5% O_2) for 7 d followed by normoxia (21% O_2) for 7 d (HN) or normoxia for 14 d (NN). The HN group showed a significantly higher amount of calcium content compared with the NN group. $^aP < 0.05$.

increased osteogenic ability of the cells in the HN group in our study also developed through endochondral ossification. However, the time course is relatively short and additional experiments will be needed to confirm this hypothesis.

There were a few limitations in this study. First, because we used only rat MSCs, evaluations using human MSCs should be performed in future experiments. Second, more detailed intracellular signaling changes caused by the hypoxic environment should be elucidated at the molecular level. Third, although the temporal combination of hypoxia and normoxia clearly boosted osteogenesis of rat MSCs, it is unclear whether this increased osteogenesis is solely caused

by the increase in cell number or whether hypoxic preconditioning also enhanced the differentiation and mineralization of these cells. Despite these limitations, our findings offer an important insight into the potential for hypoxic preconditioning in the field of bone regenerative medicine.

In conclusion, we show that modifying oxygen tension can improve the higher osteogenic ability of rat MSCs both under *in vitro* and *in vivo* conditions. Thus, hypoxic preconditioning appears to be an effective method for MSC-mediated bone tissue engineering.

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COMMENTS

Background

Bone tissue engineering using marrow stromal cells (MSCs) as a cell source has been widely studied. Among several factors affecting MSC osteogenesis, oxygen tension is important; however, the specific tension and timing of hypoxic preconditioning remains controversial. Here, the authors investigated how hypoxia affects the osteogenic ability of rat MSCs *in vitro* and within cell sheets in a subcutaneous scaffold *in vivo*.

Research frontiers

Various methods have been reported to enhance the osteogenic ability of MSCs, including the use of scaffolds and growth factors. However, considering the potential disadvantages of these approaches, such as immunological responses to the scaffold, as well as the complicated procedure of producing suitable scaffolds for insertion, techniques that avoid these approaches would be better suited. In this context, the authors suggest that modulating oxygen

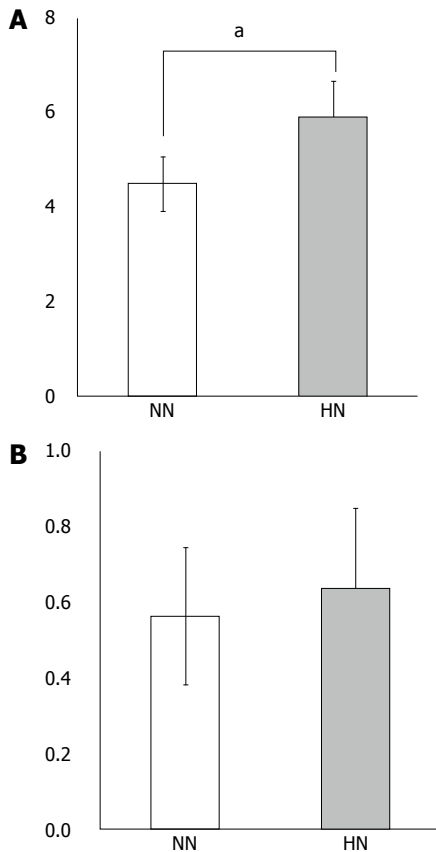


Figure 8 Real-time quantitative PCR of β -tricalcium phosphate discs wrapped with osteogenic matrix cell sheet. Increased collagen type I mRNA expression was observed in the HN group exposed to hypoxia (5% O_2) for 7 d followed by normoxia (21% O_2) for 7 d, compared with the NN group exposed to normoxia for 14 d (A). A tendency for higher expression of osteocalcin mRNA was also observed in the HN group (B). $^aP < 0.05$.

tension and the use of osteogenic matrix cell sheet (OMCS) could offer a promising method for bone repair.

Innovations and breakthroughs

Here, the authors show that modifying oxygen tension using hypoxia preconditioning can enhance the osteogenic ability of MSCs *in vitro*, and similarly advance bone formation in OMCS wrapped around scaffolds when implanted subcutaneously on the backs of rats.

Applications

Modulating oxygen tension to enhance osteogenesis is a simple and inexpensive preconditioning method. Combining OMCSs with appropriately timed hypoxia can enhance bone tissue engineering.

Terminology

MSCs: Marrow stromal cells, derived from the bone marrow. MSCs can be induced to differentiate into osteogenic, chondrogenic, adipogenic or other cell lineages with the appropriate media conditions. These cells are routinely used as a cell source for musculoskeletal tissue engineering purposes. OMCSs: Osteogenic matrix cell sheets are MSCs cultured with dexamethasone and ascorbic acid phosphate (originally with also β -glycerophosphate). The cells undergo differentiation and matrix production, producing a cell sheet structure that can be collected as a single cell sheet. These sheets offer *in vitro* osteogenic potential and *in vivo* bone formation without the need for scaffolds. OMCSs in the current study were implanted into the backs of rats using a β -TCP scaffold for positioning and to be able to identify the OMCS later for harvesting to measure bone formation changes. OMCSs can be used alone for non-union surgery and to accelerate bone-tendon healing in ligament reconstruction.

Peer-review

This study is new. This manuscript aims at investigating whether modifying oxygen tension affected MSC osteogenesis. The authors found that low oxygen pretreatment for 7-d following by 7-d treatment under normal oxygen could promote MSCs' osteogenic differentiation in both *in vitro* and *in vivo* models.

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Adipose-derived stromal cell in regenerative medicine: A review

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Abstract

The application of appropriate cell origin for utilizing in

regenerative medicine is the major issue. Various kinds of stem cells have been used for the tissue engineering and regenerative medicine. Such as, several stromal cells have been employed as treat option for regenerative medicine. For example, human bone marrow-derived stromal cells and adipose-derived stromal cells (ADSCs) are used in cell-based therapy. Data relating to the stem cell therapy and processes associated with ADSC has developed remarkably in the past 10 years. As medical options, both the stromal vascular and ADSC suggests good opportunity as marvelous cell-based therapeutics. The some biological features are the main factors that impact the regenerative activity of ADSCs, including the modulation of the cellular immune system properties and secretion of bioactive proteins such as cytokines, chemokines and growth factors, as well as their intrinsic anti-ulcer and anti-inflammatory potential. A variety of diseases have been treated by ADSCs, and it is not surprising that there has been great interest in the possibility that ADSCs might be used as therapeutic strategy to improve a wider range of diseases. This is especially important when it is remembered that routine therapeutic methods are not completely effective in treat of diseases. Here, it was discuss about applications of ADSC to colitis, liver failure, diabetes mellitus, multiple sclerosis, orthopaedic disorders, hair loss, fertility problems, and salivary gland damage.

Key words: Adipose-derived stromal cell; Colitis disease; Liver failure; Diabetes mellitus; Multiple sclerosis; Orthopedic disorders; Hair loss; Fertility problems; Salivary gland damage

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Core tip: Nowadays, adipose-derived stromal cells (ADSCs) are one of the most important and promising cell sources in the field of regenerative medicine. Unique capabilities of ADSCs caused them to be used in both research and treatment as a valuable resource in basic science and medical researches. In over 15 years

since their discovery, ADSCs have transformed our toolkit for treating human disorder and disease. As the field enters its next decade, a new wave of therapeutic applications, such as hepatic regeneration, diabetes mellitus treatment, multiple sclerosis treatment, and orthopaedic disorders regeneration, has converged with ADSCs to yield new insights for their use in stem cell engineering and regenerative medicine.

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INTRODUCTION

Mesenchymal stromal cells (MSCs) are undifferentiated cells that are able to renew their population and become differentiated to produce all specialized cell types of the tissue from which they are originated^[1] (Figure 1). While MSCs are traditionally isolated from bone marrow, over the last few years, they have also been found in many other adult tissues such as liver, cord blood, placenta, dental pulp and adipose tissue^[1]. The different stromal cells have some features in common, including morphological and immunophenotypic properties^[1]. Although, bone marrow-derived stromal cells (BMSCs) and adipose-derived stromal cells (ADSCs) are better known than others^[2-4]. ADSCs share biological properties with stromal cells obtained from bone marrow; however, these candidate cells also have some different properties compared to BMSCs^[2,3].

Furthermore, Both Adipose-derived stromal cells and bone marrow-derived stromal cells have played a prominent role in regenerating the defective tissue of patients^[4]. In the recent years, as one of the most successfully developed stem cells, adipose-derived stromal cell is a better choice than many other adult stem cells such as bone marrow-derived stromal cell because of its characters^[4].

Such as, ADSCs not only have decreased sampling risk for individual donors compared with BMSCs but also have been needed to an easier method for isolation compared with BMSCs^[4]. The adipose tissue, ADSCs' harvested source, could also provide a higher number of stromal cells compared with bone marrow tissue as BMSCs' obtained source^[4]. Furthermore, ADSCs are superior to BMSCs in some biological features, including the immune feature regulation^[4]. In addition, with an emphasis on adult stem cells rather than on embryonic stem cells, regenerative medicine programs are using ADSCs as more applicable adult stem cells to treat different diseases^[4]. Today, Adipose-derived stromal cells are known as a rich source of MSCs which are considered a suitable case for repair and regeneration of various tissues because of their rapid proliferation

and multilineage potential^[2-5]. Several properties making scientists to pay attention to ADSCs include Immunomodulatory effects and secretion of a variety of growth factors and cytokine as well as anti-apoptosis and anti-inflammation potential^[6]. *In vitro* ADSCs are identified by plastic adherence, colony forming capacity, rapid proliferation and lack of major histocompatibility class II (MHC II)^[7,8]. ADSCs not only are interesting in basic sciences, but also have been used in a broad range of regenerative medicine application, such as orthopaedic damage, fertility problems, hair loss, Colitis disease, liver failure, diabetes mellitus, multiple sclerosis, etc. In the treatment of many of different diseases, ADSCs have exhibited a great potential for tissue repair and modulation of host immune response *in vivo*^[6]. ADSCs from healthy donors are an attractive cell source for organ regeneration^[9]. These cells can be obtained and cultured *in vitro* in sufficient numbers and subsequently used in damaged tissue regeneration^[10]. So far it has been well recognized that these cells possess a broad spectrum of differentiated potentials, from cell types of mesodermal origin to ectoderm (such as hepatocyte) and endoderm (such as beta cells), when induced *in vitro*^[10-13] (Figure 2). ADSC can be expanded effortlessly in culture for long periods of time without losing their differentiation capacity^[12]. They are robust cells, which can easily survive freezing temperatures with limited loss in viability, proliferative capacity and differentiate potency^[12]. The most attractive aspect of ADSCs is their immunosuppressive properties that allow transplanting them irrespective of a human leukocyte antigen (HLA) match between the host and the donor^[14]. These cells are negative for surface marker proteins, such as CD14, CD34, and CD45, *in vitro*^[15]. Although, these candidate cells express CD34 *in vivo*^[16].

Moreover, ADSCs express cell surface markers, including CD10, CD13, CD29, CD44, CD71, CD73, CD90, CD105, CD166 and CD271 (Figure 3) and different varieties of trophic factors, such as molecular regulation of cell growth and proliferation, fibrosis, angiogenesis, and immune suppression^[7,17-24]. Additionally, the Anti-apoptotic, anti-oxidant, anti-inflammatory activities of the ADSCs are among other important characteristics that can affect their regenerative potential^[9,25-29].

Furthermore, ADSC treatment is now a widely used therapeutic strategy in the field of medicine because of its intrinsic therapeutic properties, relatively easy approach to harvesting them, and the large number these cells obtained after isolation^[29].

In 160 clinical trials, ADSC-based therapy has been also used to treat various diseases such as orthopaedic disorders, hepatic failure, inflammatory diseases, and autoimmune disease^[30].

SAFETY ISSUES OF ADSC CELLS

The safety study of ADSCs conducted by the different preclinical and clinical trial has documented that these

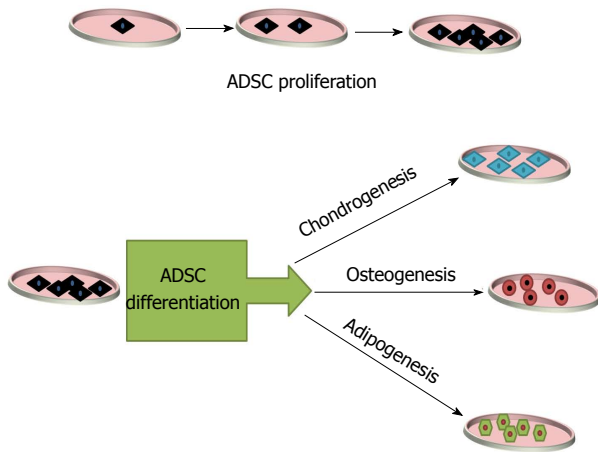


Figure 1 Schematic demonstration of the Biological Properties of human adipose-derived stromal cells. ADSC: Adipose-derived stromal cell.

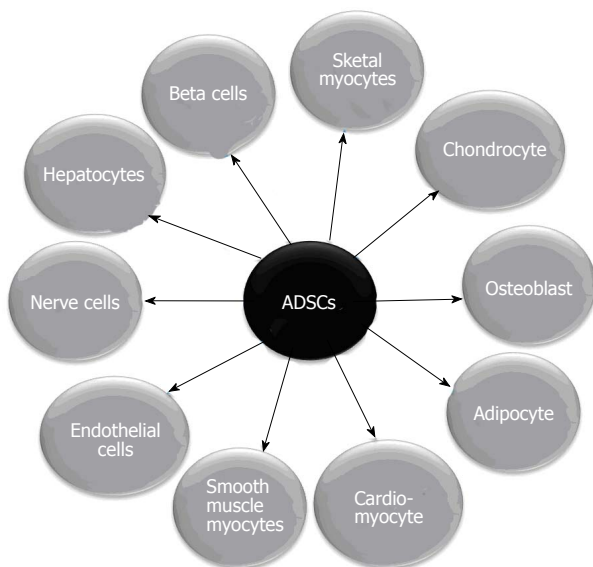


Figure 2 Schematic demonstration of the multidifferentiation potential of human adipose-derived stromal cells *in vitro*. ADSCs: Adipose-derived stromal cells.

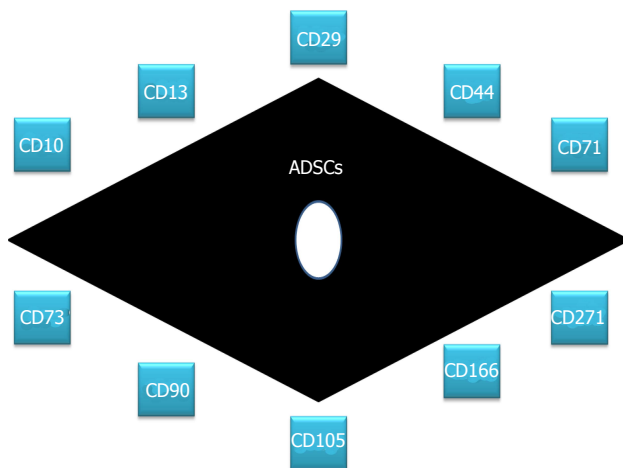


Figure 3 Schematic illustrations of the surface markers of freshly isolated human adipose-derived stromal cells. ADSCs: Adipose-derived stromal cells.

candidate cells are safe enough to be used in various treatment methods and can also play an effective role in the treatment of diseases^[31-42]. Such as, the current finding has shown that autologous ADSCs could act as a safety agent in muscle defect regeneration, both smooth and skeletal muscle, due to their profibrotic properties as well as trophic factors^[43]. Based on the results obtained from a clinical trial study, it was verified that used adipose-derived stromal cell (ADSC) implantation showed an appropriate safety feature with no serious complication in patients with degenerative disc disease^[44]. Furthermore, in phase II of the clinical trial study, the ADSC injections into the knee of 18 patients with osteoarthritis (OA) showed that these procedures do not have any severe adverse effects^[45].

However, there is little report about severe adverse effects. Such as, some of the adverse effects observed during the study include headache, inflammation, etc.^[46]. Furthermore, the safe use of ADSCs in cosmetic reconstructive surgery following a tumor is particularly doubtful because of the potential of these candidate cells to promote the development and progression of cancer^[47,48].

These cell candidates could be further assessed for understanding their therapeutic potential and safety issues in them.

In review study, we focused on ADSCs application in treat of inflammatory disease, liver failure, complication related to diabetes mellitus, multiple sclerosis diseases, orthopaedic disorders, hair loss, fertility problems, and salivary gland damage, both *in vivo* and clinical study. Also, it was provided the significant number of ADSC-based clinical trials (Table 1)^[35-41,45,49-55].

COLITIS DISEASE

Colitis, an inflammation of the colon, was treated with using intraperitoneal injection 10^5 - 10^6 human ADSCs or murine ADSCs in a study^[56,57]. This study was associated with reduced weight loss, improved survival and improved clinical in ADSC groups^[57]. In another study, intravenous tail vein administration of 10^6 macrophages cultured with either human ADSCs vs mouse ADSC lead to ameliorated disease activity index, alleviated weight loss and mortality in mice treated with ADSCs and ADSC-MF (macrophages cultured with ADSCs)^[58]. Also, intraperitoneal infusion 2×10^6 human and mouse ADSCs demonstrated significant attenuate in inflammation scores overall the colon and increase weight^[59].

LIVER FAILURE

The liver is a complicated organ that plays a metabolic function in human body. Any damage to this vital organ causes irreparable damage in the body. Due to this fact that adipose-derived stromal cells can differentiate into hepatocyte-like cells, both *in vitro* and *in vivo* condition, as well as capabilities such as homing in

Table 1 List of clinical trials that use stromal vascular fraction or adipose-derived stromal cells for a variety diseases treatment

Type of cells	Clinical trial phase	Disease	No. of patient	Highlight finding	Ref.
Autologous ADSCs	Phase I	Amyotrophic lateral sclerosis	27 patients	Low back and radicular leg pain were observed, no tumor formation were observed	[35]
Stromal vascular fraction	Phase 0 (CSN111)	Peyronie's disease	11 patients	No serious adverse events were observed	[36]
Autologous adipose-derived stromal vascular fraction	Phase I	Impaired hand function in patients with systemic sclerosis	12 patients	Four minor adverse events were observed, hand disability and pain were decreased	[54]
Autologous adipose-derived stromal vascular fraction	Phase I (NTC01813279)	Impaired hand function in patients with systemic sclerosis	12 patients	Mobility, strength and fibrosis of the hand was improved	[55]
Autologous adipose-derived stromal vascular fraction	Phase 0	Systemic sclerosis	12 patients	Finger oedema, skin sclerosis, motion and strength of the hands were ameliorated, hand pain was decreased	[49]
Stromal vascular fraction combined with PRP	Phase 0 (NCT02097862)	Degenerative disc disease	15 patients	No serious adverse events were observed	[37]
Autologous ADSCs	Phase I / II	Osteoarthritis	18 patients	Adverse events were observed in several patients, including urinary stone, arthralgia, pain and tenderness in the pes anserinus of the ipsilateral knee; ADSCs injection into the osteoarthritic knee ameliorated function and pain of the knee joint	[45]
ADSC	Phase 0 (NCT02357485)	Osteoarthritis	6 patients	No serious adverse events were observed, pain for osteoarthritis of the knee was decreased	[38]
Stromal vascular fraction containing ADSCs	Phase 0	Osteochondral lesions of the talus	49 patients	SVF Containing ADSCs administration was a therapeutically beneficial strategy for osteochondral lesions of the talus	[50]
Expanded ADSCs	Phase 0	Patients with a desire to become pregnant (with Crohn's Perianal Fistula)	6 patients	Local administration of ADSCs did not impact on course of pregnancy or newborn development;	[39]
Adipose-derived mesenchymal stem cell	Phase I / II (NCT02513238)	Salivary gland hypofunction and radiation-induced xerostomia	30 patients	No serious adverse events were observed Change in unstimulated whole salivary flow rate was observed; no serious adverse events were observed	[40]
ADSC	Phase 0	Alopecia	20 patients	Hair diameter and density were improved,the efficacy and the safety of the treatment with ADSCs was confirmed	[51]
Conditioned media of adipose tissue-derived stem cells	Phase 0	Female pattern hair loss	27 patients	Hair density was enhanced, hair thickness was improved	[52]
ADSC conditioned medium	Phase 0	Alopecia	22 patients	Hair numbers were considerably enhanced	[53]
Expanded autologous ADSC	Phase 0	Type 2 diabetes mellitus	3 patients	Blood glucose levels were decreased in all patients, no serious adverse events were observed	[41]

ADSCs: Adipose-derived stromal cells; PRP: Platelet-rich plasma.

the defect location, and immunomodulatory and anti-apoptotic mechanism, they are used for liver failure treatment^[29,60,61]. Furthermore, these cells are including anti-inflammatory factors and secrete various factors involved in tissue regeneration and are considered as a new therapeutic strategy to rebuild of liver damage^[60,62].

Previous studies have display that ADSC transplantation demonstrates appropriate therapeutic outcomes for multiple diseases, including liver failure^[63-65]. It is cleared that human ADSC transplantation could efficiently improve the liver function of acute liver failure (ALF) rats^[66]. Furthermore, ADSCs administration

increased the survival rates as well as decreased the ALF conditions in an immunocompetent ALF rat model^[67].

DIABETES MELLITUS

Diabetes mellitus, a multifactor disease, is one of the main factors of death around the world. Because of the regenerative capacity and growth factors, cytokines, and chemokines secretion, in addition to angiogenesis and vascularization features, stromal vascular fraction has suitable potential for the therapeutical application in major complication of diabetes mellitus including

foot ulcer related to diabetic, nephropathy and retinopathy^[68]. An experiment on diabetes athymic rat illustrate that ADSCs injection to vascular network of retina dysfunction site can significantly decrease apoptosis and vascular leakage and increase vascular synthesis and attenuate neurodegeneration^[69].

MULTIPLE SCLEROSIS

Multiple sclerosis, one of the most devastating autoimmune diseases of the nervous system, can be found throughout the entire world^[70]. Several animal studies have been performed on this disease using ADSC and stromal vascular fraction (SVF)^[68,71-75]. In other studies the beneficial effects of ADSC and SVF have been evaluated on experimental autoimmune encephalitis (EAE), another disease of the nervous system^[76,77]. One such animal study indicated that SVF may also have a therapeutic effect on multiple sclerosis^[76]. In another study, the use of both ADSC and SVF resulted in a reduction in the demyelination and pathological features of EAE^[78]. Both of these studies demonstrate that SVF, when employed in combination with ADSC, can lead to an amelioration of EAE in a murine model^[78]. In one study, the expression level of interleukin-10 as an immunomodulator factor was high^[78]. Additionally, an *in vivo* study identified that an ADSC-conditioned medium, along with ADSC, has both neuroprotective and immunomodulatory effects, suggesting the use of this conditioned medium as a valuable agent for treatment of EAE^[79]. Meanwhile, neither pre-clinical results nor clinical evidence have demonstrated any serious adverse effects of ADSC administration^[75-78]. In one clinical study, four patients with multiple sclerosis were treated using ADSC injection^[75]. The clinical outcome demonstrated that ADSC administration is an effective treatment strategy for patients with multiple sclerosis^[75]. Moreover, the murine EAE model has demonstrated that ADSC may be used to ameliorate motor function and decrease inflammation^[76].

Moreover, in a phase I dose-escalation safety trial noted that intrathecal treatment of autologous adipose-derived stromal cells appears safe at the tested doses in amyotrophic lateral sclerosis^[35]. Compared to use of fat transplantation, use of ADSCs in systemic sclerosis (SS) patients improved mouth functional disability, demonstrating the importance of ADSCs administration in patients suffering from SS^[80].

ORTHOPAEDIC DISORDERS

Orthopaedic disorders have been considered as leading problems in the human community.

Since ADSCs contain therapeutic properties (*i.e.*, differentiation capability into a variety of cell lineage *in vitro* as well as having immunosuppressive, osteo-inductive and anti-inflammatory features), they might be used for treatment of orthopedic major diseases such as degenerative OA^[81,82].

It was reported that ADSCs increased the expression of osteogenic genes [*i.e.*, runt related transcription factor 2 (*RUNX2*), Alkaline phosphatase, Type I collagen] and chondrogenic genes [*i.e.*, Type II collagen, SRY-box 9 (*SOX9*) and aggrecan] on biomaterials in a chondrogenic inducing medium^[83].

Previous studies showed that administration of both ADSCs and SVF in early OA is a safe and therapeutically efficient approach^[82-86].

A study on rabbit model indicated that an eight week ADSCs/hydroxyapatite implantation to critical size tibial defects could remarkably enhance mineral content and bone regeneration^[87]. Additionally, two clinical trials on bone healing illustrated that ADSCs in combination with synthetic bone graft and biomaterials may affect the regeneration, augmentation and vascularization of bone fracture^[88].

Injection of ADSCs *via* second-look arthroscopy improved cartilage regeneration and decreased pain in patients with OA^[89]. In addition, Jo *et al.*^[45] (2014) reported that the injection of 1×10^8 cell/mL ADSCs improved degenerative OA of 18 patients histologically and clinically after 6 mo of injection.

HAIR LOSS

Hair loss is one of the most crucial cosmetic challenges in both women and men these days. It's a problem for the young and old alike.

ADSCs have great potential in hair repair and regeneration, so they are an important option for hair loss treatment^[51,53,90-93]. The paracrine characteristics of ADSCs may include the specific factors released by them, including VEGF, HGF, IGF, and PDGF, which exert the specified effects on hair loss regeneration^[51-53,93-95]. These factors are too therapeutically appropriated to be used for clinical application in patients with hair loss^[51-53,93-95]. According to recent studies, it has been found that using ADSCs can stimulate hair growth in animal models^[93]. Studies have shown that the conditioned medium (CM) derived from ADSCs also had proliferative effects on hair cells *in vitro*^[91]. It was declared that a conditioned medium of ADSCs could lead to hair regeneration by promoting hypoxia^[96]. Furthermore, a clinical trial involving 22 participants with alopecia documented that intradermal administration of a conditioned medium of ADSCs may lead to an ameliorating effect in hair regeneration process^[53].

These medium candidates, in combination with LL-37, could also induce hair regeneration *in vivo*^[90]. Furthermore, a study demonstrated that ADSCs-conditioned medium not only has a stimulated alkaline phosphatase activity, but is also related to dermal papillae cells and dermal papillae markers^[97].

In addition, a retrospective observational study noted that hair density and thickness could be improved following 12 wk of CM-ADSC administration^[52].

Previous evidence has demonstrated that ADSCs and adipocytes could act as a niche for hair follicles,

due to providing an increase in the skin's thickness and progress in the intradermal adipocyte layer during the anagen phase, as well as creating a decrease in the intradermal adipocyte layer during the catagen and telogen phases^[98,99]. In addition, ADSCs and adipocytes regulate the hair cycle *via* the release of signaling molecules, *i.e.*, WNTs, PDGF, BMPs, and FGFs^[98,99]. These signals could lead to activation of the stem cell differentiation in the hair follicle and bulge stem cell activation during the telogen phase^[98,99]. Canine ADSCs administration could also be caused by the increase in the vascularization process in the dermal papillae and has a beneficial effect on hair growth and repair in the nude mice model^[100].

In addition, an animal study showed that ADSCs in combination with core-shell sphere could help in the formation of hair^[97].

ADSCs can also support auditory hair cells, and these cells are capable of regenerating damaged hair^[101].

Furthermore, protein secreted by ADSCs may be considered an appropriate tool for hair repair^[93]. Such as, an observational pilot experiment performed on twenty seven patients with female pattern hair loss demonstrated that administration of protein extract derived from ADSCs could be caused to enhance in hair density and thickness since 12-wk follow up treatment. Furthermore, no serious complication was observed in patients^[93].

Similarly, another pilot experiment verified the beneficial effect of ADSCs protein extract in patients with male pattern hair loss^[93].

FERTILITY PROBLEMS

Infertility is one of the most common problems impacting both men and women. This health issue could lead to decreased populations, and treatment strategies are necessary to address it. However, many current therapeutic strategies are not very effective. As a result, more efficient treatments should be developed. One such solution is ADSCs-based therapy, which has been demonstrated to lead to improvements in fertility rates.

An animal study illustrated that the administration of ADSC could be considered as a therapeutic strategy in chemotherapy-induced ovarian dysfunction in rat models^[102].

For example, the use of ADSCs caused a significant increase in the number of maturing follicles and corpora lutea with definite oocytes inside^[103].

In another preclinical experiment, the use of collagen scaffold in combination with ADSCs enhances the short-term maintenance of ADSCs in ovaries^[104].

It aims for long-term recovery of ovarian function, in addition to improving the fertility of rats with premature ovarian failure^[104].

Similarly, Sun *et al.*^[105] (2013) demonstrated that intraperitoneal injection of ADSCs could ameliorate ovarian function in mice with chemotherapy-induced

ovary damage.

Furthermore, on considering an *in vitro* study, it was identified that conditioned medium obtained from ADSCs could lead to human oocyte maturation and embryo formation following intracytoplasmic sperm injection through secretion of paracrine factors^[106].

It is elucidated that the administration of ADSCs could promote fertility restoration in azoospermia rats, as well as the generation of sperm in them^[107].

In addition, a human *in vitro* study reported that supernatant product of ADSCs (SPAS) could be ameliorate sperm motility in male infertile patients that can be due to existence of bioactive molecules and growth factors, which have a positive effect on sperm motility parameter^[108].

In several animal studies, it was identified that ADSCs injection or transplantation have a positive impact on the viability of ovarian follicles and could increase the retention of short-term cryopreserved ovarian grafts, as well as improve the graft quality in the rat model^[102-105,109].

Several preclinical and *in vivo* experiments have verified that the administration of ADSCs may have a beneficial effect on Peyronie's disease, which is a problem that could lead to infertility^[110-113].

Considering obtaining data from a pilot study, the application of autologous SVF in combination with shock wave may have a therapeutic effect on Peyronie's disease^[36].

In addition, this study on 11 patients documented that the administration of these adult stem cells is a safe process for the treatment of Peyronie's disease^[36].

SALIVARY GLAND DAMAGE

The salivary gland is considered as one of the most exocrine glands that generate saliva, which helps the chewing and swallowing process.

Radiotherapy is one of the most well-known agents that may cause damage to the salivary gland.

Due to their capacity to differentiate into salivary gland cells and their potential to secrete bioactive molecules, as well as their capability to induce regeneration following salivary gland failure, ADSCs have been considered promising tools for salivary gland damage regeneration^[114-124].

There are a number of researches and clinical studies which have looked into salivary gland damage regeneration through ADSC application, by means of local and systemic use^[114-124].

It was demonstrated that systemic use of ADSCs could provide a support against salivary gland damage induced by irradiation^[114]. In addition, it was identified that ADSCs may migrate engrafting to an injured location *via* the blood stream^[114,120,121].

Similarly, Maria *et al.*^[119] (2011) documented that ADSCs non-permanently supply a salivary gland cell of the endothelial or salivary acinar cell phenotypic trait by transdifferentiation into salivary gland cells.

It was identified that ADSC administration may be decreased in the apoptosis process through secreted growth factor with anti-apoptotic action^[114,116,117,120,122]. Furthermore, the fibrosis reaction was diminished after ADSC administration^[114,120]. It has been proposed that a paracrine mechanism could be responsible for the improvement of induced damage by radiation through providing growth factors related to neo-vascularization^[120].

Both animal and human experiments have verified that ADSCs could represent a safe treatment strategy for salivary gland damage^[40,114,115,117,120,123].

In addition, secreted bioactive factors from ADSCs could promote epithelial proliferation and a stimulated angiogenesis process^[114,120,124].

One study explored the local administration of ADSCs for improved tissue remodeling effectiveness in impaired salivary glands induced by radiation^[117]. This study showed that ADSCs could lead to beneficial results by ameliorating the tissue remodeling of impaired salivary glands induced by radiation^[117].

Furthermore, it was elucidated that ADSC secretome from a hypoxic-conditioned medium may provide a positive outcome on radiation damaged salivary glands, as well as supplying ameliorating and remodeling effects on damaged tissue by paracrine mechanisms^[118].

It was noted that ADSC application could also amend xerostomia induced by radiation, a problem related to salivary dysfunction that it is created following radiotherapy for head and neck cancer, through high expression of a variety of growth factors, including hepatocyte growth factor, and vascular endothelial growth factor^[40,120,121].

CONCLUSION

Cell-based therapy has been used during the recent years to treat a variety of body damages and lesions. A variety of stromal stem cells harvested from several different tissue types have therapeutic characteristics, but BMSCs and ADSCs are widely considered more usable candidates for regenerative medicine among them. The application of ADSCs is greater than that of BMSCs in regenerative medicine because ADSCs have need to more easily technique for isolation compared to BMSCs, as well as they have a much greater rate in number than to BMSCs. because the technique for isolating ADSCs is easier and, consequently, they can be used in greater number than BMSCs.

As a result of their inherent therapeutic properties, ADSCs could also provide a hopeful strategy in the field of regenerative medicine for treatment a wide range of diseases and lesions. This will ensure the availability of ADSCs for research, trial and clinical applications in the future. Due to the promising results obtained from preclinical and clinical trials as well as their unique features in term of regenerative potential, these cells can be useful in the treatment of different diseases. Furthermore, it has been shown that the administration

of ADSCs can provide a safe treatment strategy in regenerative medicine approaches. There have been few reported serious side effects resulting from the clinical use of ADSCs, although there have been some reports concerned with adverse effects. Such limited adverse effects observed in some trial studies include headache, inflammation and etc. additionally, considering to previous data, ADSC promote carcinoma progression and for that reason appear to increase the risk of cancer relapse in breast augmentation procedures. Therefore, there are needs for further research on understanding the potential application of ADSC as a safe and effective therapeutic option on diseases treatment in future.

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Epithelial plasticity and cancer stem cells: Major mechanisms of cancer pathogenesis and therapy resistance

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Abstract

Epithelial-mesenchymal transition (EMT) has been linked with aggressive tumor biology and therapy resistance. It plays central role not only in the generation of cancer stem cells (CSCs) but also direct them across the multiple organ systems to promote tumor recurrence and metastasis. CSCs are reported to express stem cell genes as well as specific cell surface

markers and allow aberrant differentiation of progenies. It facilitates cancer cells to leave primary tumor, acquire migratory characteristics, grow into new environment and develop radio-chemo-resistance. Based on the current information, present review discusses and summarizes the recent advancements on the molecular mechanisms that derive epithelial plasticity and its major role in generating a subset of tumor cells with stemness properties and pathophysiological spread of tumor. This paper further highlights the critical need to examine the regulation of EMT and CSC pathways in identifying the novel probable therapeutic targets. These improved therapeutic strategies based on the co-administration of inhibitors of EMT, CSCs as well as differentiated tumor cells may provide improved anti-neoplastic response with no tumor relapse.

Key words: Epithelial-mesenchymal transition; Anticancer therapies; Cancer stem cells; Molecular pathogenesis; Tumor relapse

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Core tip: Frequently observed reason for the failure in the treatment of malignant carcinomas is the biological programming of epithelial cells called epithelial-mesenchymal transition (EMT). It confers cancer cells, an ability to lose epithelial traits; gain mesenchymal traits; acquire stem-like properties; disseminate and colonize to distant organ sites and show elevated resistance to cancer therapies. Partial elimination of cancer stem cells and their propagation into secondary tumors post-treatment are the limitations associated with currently available standard of care including radio/chemotherapies, surgical resection or combination of these. Differentiation-based therapeutic strategies utilize the variable and regulatory powers of EMT program, lead to successful eradication of stem-like population of cancer cells by reverting the EMT phenotype and may hold great promise in improving the clinical outcomes.

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INTRODUCTION

Despite significant advancements in standard treatment modalities including surgical resection, radio and chemotherapeutic procedures to treat cancer, there has been a tremendous increase in cancer related deaths globally. Although current therapeutic strategies have been successfully implicated and resulted improved tolerance and organ preservation in patients with locally advanced cancer but fail to prevent tumor from relapse. Therapeutic resistance leads to cancer pathogenesis, tumor recurrence and metastasis.

Accumulation of multiple genomic mutations in cells leads to genetic instability or oncogene-induced plasticity. Genetic and epigenetic changes may transform normal stem cells, differentiated cells or progenitor cells into cancer stem cells (CSCs) and allow the development of tumors. Existence of quiescent CSCs has been reported during intense growth of tumor and examined to possess the potential to self-renew, ability to proliferate and aberrantly differentiate into heterogeneous lineages of cancer cells. Cancer cells leave primary tumor site, extravasate to distant organs through blood or lymphatic system, colonize into new environment and develop resistance to therapeutic drugs.

Dissolution of intercellular adhesions and loss of epithelial polarity as a result of epithelial-mesenchymal transition (EMT) program has been associated with uncontrolled proliferation of cells and malignant progression. EMT regulates the apico-basal polarity of epithelial cells and turns them into cells with mesenchymal traits. Eventually cancer cells proliferate extensively, invade, acquire migratory capabilities and metastasize. Altered epithelial functions enable the CSCs to survive and exhibit resistance to growth inhibitory drugs thereby contributes to long term tumor recurrence and cancer progression.

Understanding the molecular mechanisms that control cancer pathobiology and therapeutic resistance may allow us to identify the biomarkers of potential clinical significance and novel therapeutic targets to treat cancer for their effective eradication and improved clinical outcomes. This paper summarizes the major findings on mechanistic regulation of EMT that transforms stem cells into CSCs, its major functions in metastatic activities, drug resistance and therapeutic implications.

EPITHELIAL PLASTICITY

EMT program is classified as EMT type 1, EMT type 2 and

EMT type 3 and considered as an important physiological phenomenon in organogenesis during embryonic development, wound healing and cancer respectively^[1]. It is characterized by the chain of events that starts from cells' inside to extracellular matrix (ECM) and includes loss of epithelial cell-cell junctions and cell polarity, stress fiber redistribution and transition from epithelial phenotype to mesenchymal (fibroblastic) phenotype. Epithelial tumor cells exhibit cellular plasticity, undergo transition from epithelial (E) to mesenchymal (M) phenotype, dismantle basement membrane, infiltrate the surrounding tissues and metastasize to distant sites. This is followed by the growth of secondary tumors and regain of epithelial characteristics, required during differentiation through the activation of reverse program-mesenchymal to epithelial transition (MET)^[2] (Figure 1).

Cellular dissociation, morphological change to more prolonged forms and increased migration abilities of cells are described by molecular characteristics which include increased expression of N-cadherin, vimentin, type 1 collagen, β -catenin stabilization, repression of E-cadherin, claudins, zona occludens 1, occludins, cytokeratins, basement membrane components collagen IV and laminin 1 and release of matrix remodeling enzymes (matrix metalloproteinases). Molecular changes in the basic foundations in epithelial architecture are noted as a result of induction of EMT-activating transcription factors (EMT-ATFs) such as two-handed zinc-finger factors of d-crystallin/E2 box factor 1 (dEF1) family proteins, EF1/ZEB1 [dEF1/zinc-finger E-box-binding homeobox 1 (ZEB1)] and SIP1 (Smad-interacting protein)/ZEB2, Snail family of zinc-finger transcription factors, Snail1 (Snail), Snail2 (Slug) and Snail 3 (Smuc), basic helix-loop-helix factors, Twist and E12/E47. Wnt, smad3 dependent transforming factor-beta (TGF- β), Hedgehog, Notch, fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin growth factor (IGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), estrogens, sonic hedgehog (Shh), and nuclear factor- κ B (NF- κ B) signaling pathways are regulated by micro-environmental stimuli and act as EMT inducers^[3,4] (Figure 2). Besides the cooperation between signaling pathways through autocrine signaling loops, inflammatory cytokines, hypoxic or oncogenic signals also contribute to EMT during cancer progression.

Change in the expression of several microRNAs (miRNAs) has been observed during induction of EMT or MET. MicroRNAs are 21-23 nucleotide long non-encoding RNA molecules, modulate gene expression post-transcriptionally and act as master regulator in many pathological and physiological processes including tumor development. Suppression of E-cadherin through direct targeting of its transcriptional repressors and hence inhibition of EMT has been associated with the expression of miR-200 family, miR-205, miR-34 family^[5-7]. Interaction of miRs with EMT-ATFs forms mutually exclusive inhibitory feedback loop and is responsible for bistable switch between E (miR-200^{high}; miR-34^{high}; Zeb^{low}; and Snail^{low})

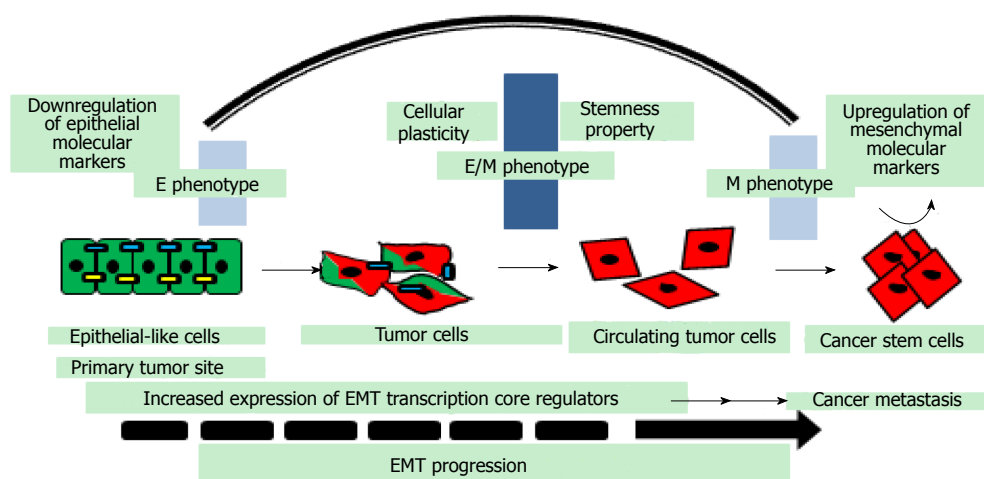


Figure 1 Epithelial-mesenchymal transition progression in epithelial cancer cells: Cancer cells with E phenotype exhibit epithelial-mesenchymal transition at primary tumor site, loose cell-cell contacts, gain migratory abilities, undergo morphological change and acquire M phenotype. Co-expression of epithelial and mesenchymal marker proteins in cancer cells with partial E/M hybrid phenotype is associated with increased cellular plasticity and stemness. Cancer stem cells with hybrid E/M phenotype undergoing partial EMT and not complete EMT gain self-renewability, migratory and invasive traits during cancer metastasis. EMT: Epithelial-mesenchymal transition; E: Epithelial; M: Mesenchymal.

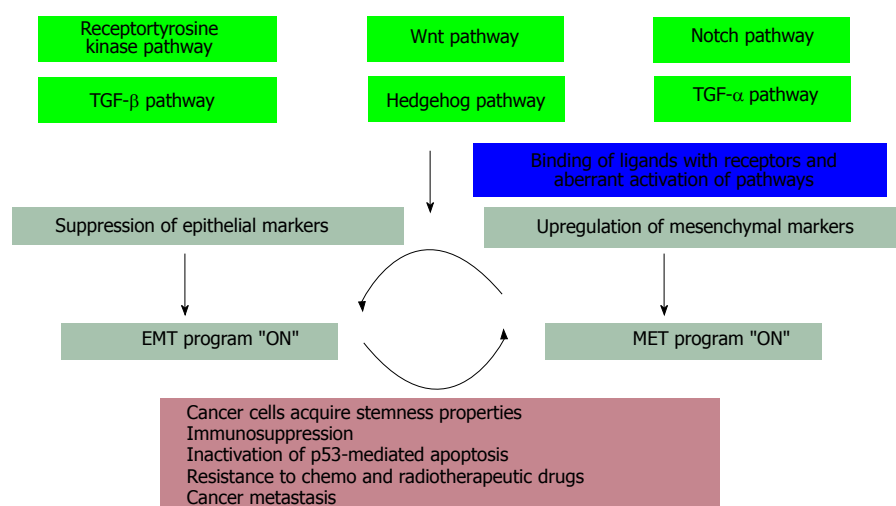


Figure 2 Signaling pathways regulating epithelial-mesenchymal transition and mesenchymal-epithelial transition: Aberrant activation of signaling pathways including Notch, Wnt, Hedgehog, receptor tyrosine kinase, Transforming growth factor-beta, tumor necrosis factor-alpha regulate the expression of epithelial-mesenchymal transition-activating transcription factors. EMT-ATFs induce EMT by repressing and activating the expression of epithelial and mesenchymal genes respectively. Epithelial plasticity confers long term survival advantages to the disseminated cancer stem cells at distant sites, makes them resistant to conventional therapies and allows the cancer to relapse. EMT: Epithelial-mesenchymal transition; MET: Mesenchymal-epithelial transition; TGF-β: Transforming growth factor-beta; TNF-α: Tumor necrosis factor-alpha; EMT-ATFs: EMT-activating transcription factors.

and M (miR-200^{low}; miR-34^{low}; Zeb^{high}; and Snail^{high}) phenotypes^[8] (Figure 3).

As per the tumor progression model described by Brabletz *et al*^[9], migrating CSCs with stem-like characteristics at tumor-host interface acquire migratory capacities through EMT and are responsible for the formation of primary tumor, metastatic dissemination of cancer cells, recurrence and therapeutic resistance.

CSCs

Theory of CSCs and the fact that cancer arises from the rare subset of cells with stemness properties was

conceptualized around 150 years ago. Small population of tumor cells expressing specific surface markers including CD34⁺ and CD38⁻ in human acute myeloid leukemia has been identified as CSCs or cancer initiating cells (CICs) by Bonnet and Dick^[10]. These cancer cells were later described in other tumors including head and neck, breast, prostate, lung, liver, pancreas, colon and bladder. CSCs and stem cells share similarities in terms of expression of specific surface markers, regulation by stem cell niche and signaling pathways as well as their self-renewal abilities. Tumorigenic activities exhibited by CSCs allow them to differ significantly from normal stem cells. Studies from hematological

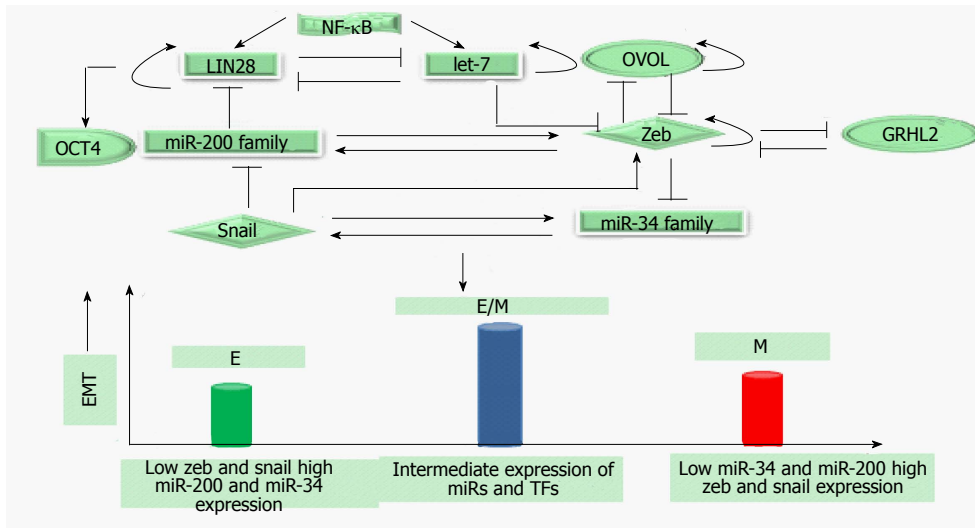


Figure 3 Epithelial-mesenchymal transition regulatory network: Mutually exclusive inhibitory loops including miR-200family/Zeb; miR-34family/Snail; LIN28/let-7 bring about bistable switch between epithelial (E) and mesenchymal (M) phenotypes, control Epithelial-mesenchymal transition/mesenchymal-epithelial transition and stemness. Phenotypic stability factors like OVOL and GRHL2 couple to core-EMT decision making circuits and stabilize hybrid E/M phenotype. NF- κ B controls LIN28/let-7 regulation and elevates the likelihood of hybrid E/M phenotype. Solid arrows represent the activation; solid lines represent the repression and circular loops represent the self-activation. Hybrid E/M: Hybrid epithelial/mesenchymal; NF- κ B: Nuclear factor kappa B; miR: MicroRNA; EMT: Epithelial-mesenchymal transition.

as well as solid organ malignancies characterize CSCs with the ability to mediate angiogenesis, develop tumor upon their serial transplantation into immunodeficient mice and tumorsphere formation in non-adherent 3D cultures^[11,12]. The other hallmark feature of CSCs reported is aberrant differentiation where these cells undergo asymmetrical division to produce non-tumorigenic population of cells and symmetrical division to develop tumorigenic daughter cells.

Regulation of CSCs, stemness properties and their enhanced migratory characteristics has been shown to be orchestrated by the interplay of complex pathways and various transcription factors. Hedgehog, Notch, Wnt/beta-catenin, octamer-binding transcription factor 4 (Oct4), SRY (sex-determining region Y)-box 2 (Sox2) and Kruppel-like factor 4 (Klf4), high-mobility group AT-hook 2 (HMGA2), Nanog, Nestin, Bcl-2, Bmi-1, c-Myc, and c-Met are required for the differentiated cells to reprogram to pluripotent stem cells, drive the production and maintenance of pluripotent cells with stemness properties^[13-15]. Crosstalk between pleiotropically acting molecules, EMT associated genes and transcriptional mediators such as Snail/ Twist or TGF- β treatment has been linked with expression of tumor cells with CD44⁺/CD24⁻ surface markers, enhanced mammosphere formation and induction of EMT in immortalized human mammary epithelial cells^[16]. Transfection of ovarian cancer cells with EMT-ATFs Snail/Snail 2 results in derepression of stemness genes including Nanog and KLF4, allows cells to acquire CD44^{high}/CD117^{high} stem cell profile and induces EMT in more-differentiated cells to generate cells with CSC phenotype^[17].

Experimental studies underline the important functions of EMT in the generation of CSCs and conferring self-renewal ability to the differentiated tumor cells. Transition

from E to M phenotype contributes to the enhanced migration of CSCs, their dissemination in the circulation and colonization to a particular site. Thus migratory CSCs form secondary metastatic nodules and exhibit E phenotype *via* MET^[18,19].

EMT gradient model describes the bimodal nature of EMT program in epithelial cells. Cancer cells with E phenotype display stemness properties during early stage of tumor development but lose it when they acquire M phenotype. Nevertheless robust association of hybrid E/M phenotype of cancer cells that co-express epithelial and mesenchymal markers through the partial activation of EMT program has been examined with increased stemness, plasticity, self-renewability, migration capabilities and poor cancer outcomes (Figure 1). Role of phenotypic stability factors (PSFs) including OVOL and GRHL2 has been characterized in stabilization of E/M hybrid state when coupled with miR200/Zeb (EMT-decision making circuit)^[20]. miR-200 by inhibiting LIN28; NF- κ B, but not c-Myc by regulating LIN28/let-7; and OVOL by coupling with miR200/ZEB/LIN28/let-7 circuit have been examined to increase the stemness of the hybrid E/M phenotype^[21,22] (Figure 3).

Breast CSCs with E/M hybrid behavior are examined to show increased ALDH1⁺ (aldehyde dehydrogenase 1) activity, mammosphere formation, self-renewal capability and stemness as compared to highly differentiated M cells that exhibit less cellular plasticity and E cells which show less self-renewability^[23,24]. Subset of ovarian cancer cells with hybrid E/M state has been identified with low membranous and high cytoplasmic E-cadherin, high CD133, high CD44, low Tie2 expression, increased plasticity and *in vivo* xenograft tumor growth upon their transformation^[25]. Epithelial plasticity thereby facilitates metastasis formation, confers long term survival advan-

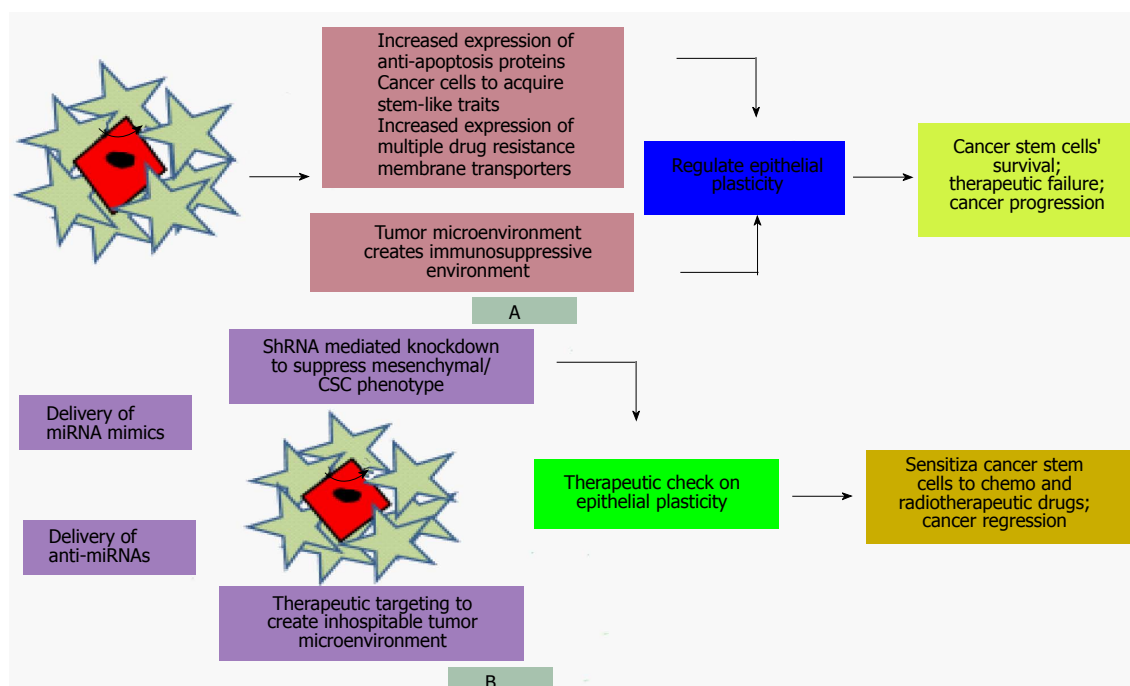


Figure 4 Cancer stem cells, epithelial plasticity and therapeutic strategies. A: Existence of quiescent CSCs that possess the potential to self-renew, ability to proliferate and aberrantly differentiate into heterogeneous lineages of cancer cells and tumor microenvironment by creating immunosuppressive environment regulate epithelial plasticity and enable CSCs to survive, exhibit resistance to growth inhibitory drugs and cause tumor to progress; B: Therapeutic strategies including delivery of miRNA mimics to enforce the expression of tumor suppressor genes, administration of anti-miRNAs to downregulate the expression of oncogenes, shRNA mediated knockdown of oncogenic factors to revert the mesenchymal/CSC phenotype to epithelial non-CSC phenotype and creating inhospitable tumor microenvironment not only confer therapeutic check on epithelial plasticity but also sensitize cancer stem cell populations to the killing effects of therapeutic drugs. CSC: Cancer stem cell; miRNAs: MicroRNAs; ShRNA: Short hairpin RNA.

tages to the disseminated cancer cells at distant sites, makes tumor cells resistant to conventional therapies and allows the cancer to relapse.

THERAPEUTIC IMPLICATIONS

Chemotherapy and radiotherapy as non-invasive as well as surgical resection or the combination of these are the most commonly used cancer therapies in clinics. These therapies although can be employed to kill bulk of the tumor and provide maximal benefit to the overall survival of the patients, nevertheless, therapies have always been associated with systemic or local toxicity, aggressive cancer relapse and drug resistance. Population of pancreatic cancer cells exhibiting resistance to gemcitabine, ovarian carcinoma cells to paclitaxel, breast cancer cells to tamoxifen or lapatinib, lung cancer cells to gefitinib have been identified with the co-existence of subset of cancer cells, CSCs with mesenchymal traits and multiple resistant mechanisms associated with them^[26-28]. Relative dormant behavior, high expression of anti-apoptosis proteins and multiple drug resistance membrane transporters, epithelial plasticity, hypoxia are some of the potential reasons of CSCs' survival and therapeutic failure. Use of combinational approaches to target EMT which is responsible for the survival of CSCs and their tumor functions offers new possible strategy for cancer therapy. Multiple powers of EMT have crystal-

lized an emerging concept of differentiation based cancer therapies as attractive targets for therapeutic intervention (Figure 4).

Prevention of STAT3-mediated transcription of ZEB1, SNAI1 *via* suppression of JAK1/2 by ruxolitinib and ZEB1 silencing through shRNA-mediated knockdown in oncostatin M (OSM, an IL-6 cytokine family member) driven mesenchymal/CSC phenotype has been examined to revert it back to an epithelial/non-CSC state in pancreatic ductal adenocarcinoma^[29]. Dai *et al*^[30] studied the therapeutic effects of ascochlorin (ASC) in increasing sensitivity to doxorubicin treatment through inhibiting STAT3 binding to the Snail promoter, reverting EMT phenotype, inhibiting metastasis in the treatment of hepatocellular carcinoma.

Delivery of anti-miR-145 using polyurethane-short branch-PEI (PU-PEI) to the mice bearing xenograft tumors has been examined to regulate Oct4/Sox2/Fascin1, Tcf4 (immunoglobulin transcription factor 4, also known as E2-2) and Wnt5a, inhibit EMT and metastatic potential and sensitize lung adenocarcinoma CSCs to chemo and radio therapeutic drugs^[31]. Forced expression of miR-200 family has been validated to restore the sensitivity of EGFR inhibitor, induces MET in mesenchymal bladder cancer cell lines, reduces tumor aggressiveness and metastatic spread^[32]. Study by Luo *et al*^[33] reports the reduced expression levels of CSCs markers LIN28B, Nanog, Oct4, and Notch1; lower expression of EMT markers MMP2, MMP3, MMP9,

Table 1 Cancer stem cells and epithelial-mesenchymal transition targeted therapy

Cancer type	Biological mechanism(s) of resistance	Targeted therapy and therapeutic functions	Clinical trial if any	Ref.
CRPC	Skp2 regulates CRPC through Twist-mediated oncogenic functions including EMT and CSCs acquisition	Genetic or pharmacological inactivation of Skp2 re-sensitize CRPC cells toward chemotherapies such as paclitaxel or doxorubicin	None	[40]
Lung cancer	High levels of circulating IGF1 lead to EMT induction and CSC maintenance	Use of IGF1R inhibitors sensitize cancer cells to killing effects of carboplatin, paclitaxel, docetaxel, and vinorelbine	Phase I trial	[41]
Ovarian cancer, advanced solid tumors	FAK linked with WNT, TGF-beta, Integrin and Hedgehog pathways, mediate cell invasion and metastasis	Anti-sense FAK oligonucleotides, adenoviral dominant-negative FAK-CD, FAK siRNA, pharmacological inhibitors affect tumor cells and microenvironment	Phase I trial	[42,43]
Invasive ductal breast cancer	Elevated expression of ABC drug transporters, induction of Wnt/ β -catenin, Hedgehog, Notch and PI3K/Akt/mTOR signaling pathways, and acquisition of EMT	Salinomycin promotes differentiation of CSCs, epithelial reprogramming of cells that had undergone EMT	Clinical pilot studies	[44]
Human lung epithelial cells	Activation of TGF- β /Smad signaling pathway, induction of EMT and therapy resistance	Use of drug, lerdelimumab, which acts as monoclonal antibody to TGF- β 1	Preclinical	[45]
Renal cell carcinoma, malignant melanoma	Activation of TGF- β /Smad signaling pathway, induction of EMT and therapy resistance	Use of drug, GC1008, which acts as monoclonal antibody to TGF- β 1	Phase I trial	[46]
Glioblastoma/anaplastic astrocytoma	Activation of TGF- β /Smad signaling pathway, induction of EMT and therapy resistance	Antisense oligodeoxynucleotide specific for the mRNA of human TGF- β 2	Phase I / II trial	[47]
Renal cell carcinoma, advanced cancers	Inflammatory cytokines including TNF- α and IL6 promote EMT and tumor invasion	Infliximab, a TNF- α monoclonal blocking antibody suppresses the levels of IL6 and CCL2	Phase II trial	[48,49]
Metastatic gastric adenocarcinoma, recurrent and metastatic head and neck squamous cell carcinoma	Activation of NF- κ B and TNF- α signaling	Bortezomib, a proteasome inhibitor suppresses NF κ B activation	Phase II trial	[50,51]
Advanced solid tumors; advanced lung cancer	Increased expression of HIF-1 α	Drug, PX-478 inhibits HIF1 α expression Topotecan along with conventional chemotherapies such as cisplatin or bevacizumab inhibit HIF-1 α expression	Phase I trial Phase I/ II trial	[52,53]

CRPC: Castration-resistant prostate cancer; Skp2: S-phase protein kinase 2; EMT: Epithelial-mesenchymal transition; CSCs: Cancer stem cells; IGF1: Insulin-like growth factor 1; FAK: Focal adhesion kinase; TGF-beta: Transforming growth factor-beta; ABC: ATP-binding cassette; siRNA: Small interfering RNA; TNF- α : Tumor necrosis factor- α ; IL6: Interleukin 6; NF- κ B: Nuclear factor- κ B; CCL2: C-C motif chemokine ligand 2; HIF-1 α : Hypoxia inducible factor 1-alpha; PX-478: S-2-amino-3-[4'-N,N-bis(chloroethyl)amino]phenyl propionic acid N-oxide dihydrochloride.

SNAIL, TWIST, Vimentin; increased expression of E-cadherin and β -catenin, and reduced sphere formation through siRNA mediated knockdown of NR5A2 (pancreatic cancer susceptibility gene) in pancreatic cancer^[33].

Clinical value of valproic acid (VPA), histone deacetylase (HDAC) inhibitor has been investigated to suppress irradiation-induced EMT, attenuate cell invasion, migration abilities and improve the effectiveness of radiotherapy in the treatment of esophageal squamous cell carcinoma (ESCC) TE9 cells^[34]. Angiopoietin-like protein 1 (ANGPTL1) has been examined to reduce EMT-driven sorafenib resistance and cancer stemness properties of hepatocellular carcinoma cells through the inactivation of MET-extracellular receptor kinase/protein kinase B- dependent early growth response protein 1-Slug (MET receptor-AKT/ERK-Egr-1-Slug) signaling cascade^[35]. Reduced expression levels of EMT-ATFs including SNAI2; self-renewal genes Nanog and BMI1; low activity of ALDH; low ratio of CDH1 (E-cadherin) and CDH2 (N-cadherin), reduced invasion and metastatic growth are the observed therapeutic effects as a result of targeting α v integrins in bladder cancer cell lines^[36].

Tumor microenvironment and its associated factors

create immunosuppressive environment, regulate the plasticity program and determine the metastatic capacity and therapeutic resistance. Prolonged stimulation of hypoxia conditions and increased hypoxia inducing factor-1 α (HIF-1 α) expression identifies and isolates breast cancer stem cells (BCSCs) with ALDH activity (CD44⁺/CD24⁻/Aldefluor^{pos}). Further knockdown of HIF-1 α has been shown to cause significant loss of stem cell properties through the reduction in the expression of mRNA genes associated with EMT (Snail, Slug and Vimentin low and E-cadherin high) and may influence breast cancer clinical outcomes^[37]. Inhibition of growth, migration and reduced radioresistance of NPCSC (nasopharyngeal carcinoma CNE-2 stem-like cell) has been observed to be a consequence of exposure of these cells with 2-Methoxyestradiol (2-ME2), a metabolic product of estrogen and X-ray. 2-ME2 has been reported to decrease NF- κ B p65 and HIF-1 α protein expression levels, downregulate NF- κ B p65 nuclear localization and reversion of EMT^[38].

Experimental studies identify novel connection between the pharmacological targeting of signaling molecules that contribute to cancer stem-like and EMT

phenotype, restrained cancer stem cell growth, inhibition of self-renewability and reduced metastatic growth *in vitro* as well as *in vivo*. Creating an inhospitable microenvironment around the protective niche of CSCs through therapeutic check on epithelial plasticity may provide the basis for developing improved therapeutic strategy in complete elimination of CSCs and bulk tumor population at primary and distant sites^[39-53] (Table 1).

Unravelling the complex interplay of molecules and understanding the functions of miRNA-mRNA interactions complex in cellular plasticity that influence the biology of CSCs, high throughput screening of drugs in combination and their clinical utility, development of effective and safe systems for the delivery of synthetic miRNA precursors in clinically relevant animal models are the major challenging issues in the development of therapeutics and their translation into clinical setting.

CONCLUSION

Frequently observed reason for the failure in the treatment of malignant carcinomas is the biological programming of epithelial cells called EMT. EMT confers cancer cells, an ability to lose epithelial traits; gain mesenchymal traits; acquire stem-like properties; disseminate and colonize to distant organ sites and show elevated resistance to cancer therapies. Aberrant activation of signaling pathways including Wnt/beta-catenin, hedgehog, Notch, receptor tyrosine kinase, TNF- α , TGF- β has critical roles in EMT. Number of experimental studies reports the regulatory effect of miRNAs on the cross talk of these pathways, EMT, generation of CSCs, cancer invasion and metastasis.

Partial elimination of CSCs and their propagation into secondary tumors posttreatment are the limitations associated with currently available standard of care including radio/chemotherapies, surgical resection or combination of these. Recent research studies come up with alternate form of therapies that can directly target, eliminate CSCs and decrease tumor relapse. Differentiation-based therapeutic strategies utilize the variable and regulatory powers of EMT program, lead to successful eradication of stem-like population of cancer cells by reverting the EMT phenotype and may hold great promise in improving the clinical outcomes.

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Basic Study

Transplanting embryonic stem cells onto damaged human corneal endothelium

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Abstract

AIM

To investigate whether human embryonic stem cells (hESCs) could be made to attach, grow and differentiate on a human Descemet's membrane (DM).

METHODS

Spontaneously differentiated hESCs were transferred onto a human corneal button with the endothelial layer removed using ocular sticks. The cells were cultured on a DM for up to 15 d. The genetically engineered hESC line expressed green fluorescent protein, which facilitated identification during the culture experiments, tissue preparation, and analysis. To detect any differentiation into human corneal endothelial-like cells, we analysed the transplanted cells by immunohistochemistry using specific antibodies.

RESULTS

We found transplanted cells form a single layer of cells with a hexagonal shape in the periphery of the DM. The majority of the cells were negative for octamer-binding transcription factor 4 but positive for paired box 6 protein, sodium potassium adenosine triphosphatase (NaKATPase), and Zona Occludens protein 1. In four of the 18 trials, the transplanted cells were found to express CK3, which indicates that the stem cells differentiated into corneal epithelial cells in these cases.

CONCLUSION

It is possible to get cells originating from hESCs to become established on a human DM, where they grow and differentiate into corneal endothelial-like cells *in vitro*.

Key words: Embryonic stem cells; Cornea; Descemet's membrane; Endothelium; Immunohistochemistry

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Core tip: This is the first report on the interaction between human embryonic stem cells (hESCs) and the inner parts of the human cornea. When hESCs were transplanted onto Descemet's membrane (DM) *in vitro* we found that they were able to attach to and grow on DM in a single cell layer. Furthermore, the stem cells changed their morphology from small round cells to flat hexagonal cells. The transplanted hESCs also started to express the proteins paired box 6, Zona Occludens protein 1 and sodium potassium adenosine triphosphatase (NaKATPase), which also are expressed in human corneal endothelial cells.

Hanson C, Arnarsson A, Hardarson T, Lindgård A, Daneshvarmaeini M, Ellerström C, Bruun A, Stenevi U. Transplanting embryonic stem cells onto damaged human corneal endothelium. *World J Stem Cells* 2017; 9(8): 127-132 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v9/i8/127.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v9.i8.127>

INTRODUCTION

The cornea is the first point of contact for visual stimuli entering the nervous system. It is responsible for approximately two-thirds of the refractive power of

the eye, and its transparency and a regular shape are pivotal for accuracy of perception. The adult cornea is made up of three cellular layers, the epithelium, stroma, and endothelium, separated by two basement membranes, Bowman's and Descemet's membrane (DM). From both a physiological and a pathological perspective, the endothelium is the most important corneal layer. The single layer of hexagonal cells that makes up the endothelium is in direct contact with the aqueous humour in the anterior segment of the eye, and preserves the transparency of the cornea by pumping water out of the stroma. Endothelial dysfunction leads to oedematous accumulation of water in the stroma, disruption of collagen fibrils, and opacification of the cornea^[1,2].

Serious corneal damage can in many cases only be treated with transplantation. Due to the global shortage of donated human tissue, there is a growing interest in developing stem cell technology as an alternative. Stem cells are undifferentiated cells that can self-renew and generate one or more differentiated daughter cells. Embryonic stem cells are considered precursor cells for all cells in the body, and therefore are ultimately the origin of all tissue. Their presence is not limited to foetal development, but is preserved along with precursor cells into adulthood. In normal or damaged adult tissue, stem cells maintain a balance between cell production and cell loss by continuously replacing older cells with newer ones. Human embryonic stem cells (hESCs) are derived from the inner cell mass of the blastocyst. They are pluripotent, which means that under certain specific circumstances they can potentially form cells of all three germ layers (ectoderm, mesoderm, and endoderm)^[3,4]. hESCs can preserve this pluripotency during long-term culture, and hence may be useful in various scientific and clinical applications^[5].

We have previously shown that it is possible to transplant pre-cultured cells derived from hESCs onto exposed (partly stripped from epithelial cells) Bowman's membrane of the human cornea^[6]. In our experiments, the transplanted cells attached efficiently to the membrane and were not detached by the sliding forces from the recipient epithelial cells during wound healing. Furthermore, one to four cell layers of epithelial-like cells were formed by the transplanted cells. Given this initial success in facilitating wound healing of the epithelium, we wanted to expand our research to include the corneal endothelium.

In the current study we attempted to get hESCs to differentiate into corneal endothelial-like cells *in vitro* on human corneas that had been partially or completely cleared of all existing endothelial cells. The overall aim was to further develop techniques for reconstructing the corneal endothelium.

MATERIALS AND METHODS

Ethical approval and samples

The study was approved by the Ethics Committee

at the Universities of Gothenburg and Malmö (approval number 067-04 and M155-14). In total we conducted experiments on 18 corneas. In all cases, undifferentiated hESCs were prepared by Collectis AB (Gothenburg, Sweden), using a protocol previously described in detail^[7]. In short, hESCs of line SA121 (Collectis, Gothenburg, Sweden)^[4] were genetically modified to constitutively express green fluorescent protein (GFP) under the human elongation factor 1- α (EF1- α) promoter. The genetically modified cell line had previously been characterized to confirm that the cells had remained pluripotent and diploid through the transfection procedures. This clone was used to facilitate the identification of the transplanted cells in the immunohistochemistry (IHC) analysis.

Cell culture and transplantation

Initially, the hESCs were cultured in Collectis DEF-Culture System (DEF-CS™; Collectis AB, Sweden), which includes neither feeder cells nor any type of membrane. Before transplantation, the homogeneously undifferentiated hESCs were allowed to initiate differentiation. For this, the cells were cultured in KnockOut™ Dulbecco's Modified Eagle's Medium (D-MEM) with 20% knock-out serum replacement (SR) and 5% foetal bovine serum (FBS) (Life Technologies Europe BV), 10 μ g/mL Hygromycin (Invitrogen, Carlsbad, CA, United States) and 5 μ mol/L of Rho-associated protein kinase (ROCK) inhibitor (Y-27632, Sigma-Aldrich, Stockholm, Sweden) for 14-22 d. On the day of the transplantation, the differentiation medium was removed and the cells were rinsed once with 1 \times phosphate buffer saline (PBS) (Ca²⁺/Mg²⁺). Subsequently, the cells were disassociated by trypsin (TrypLE™ Express, ThermoFisher Scientific Company, Waltham, MA, United States) added into the well, and incubated in 37 °C for 3 min \times 5 min. The well was gently tapped after each 5-min interval to get the cells to detach. The suspension was centrifuged and the cell pellet resuspended in 1000 μ L of the same culture medium as used for differentiation. The final cell concentration was 4 million cells/mL. About 120000 cells were put on each cornea. Human corneal tissue was obtained from patients undergoing penetrating keratoplasty for keratoconus or for corneal decompensation at Mölndal University Hospital in Gothenburg, Sweden. The removed corneal button, 7.5-8.0 mm in diameter, was kept in Modified Eagle's Medium (MEM) (Invitrogen, Paisley, United Kingdom) until the surgery was completed, and then transferred to the laboratory for further processing. Prior to each experiment, the endothelial cells were removed using an ocular stick (Pro-ophta ocular stick, Lohmann and Rauscher, Regensburg, Germany). First using the short end, all fluid was removed from the surface. Then the cells were removed with short, gentle strokes parallel to the surface. By adjusting the pressure the cells were made to detach without causing damage to the DM. Repeating this motion six to eight times removed close to all endothelial cells. The GFP-expressing cells were

cultured on the corneas for 10-15 d. The medium was changed every 2-3 d throughout the period.

Paraffin embedding and sectioning

After the 18 corneas were cultured they were fixed in 4% formaldehyde for 24 h and then placed in 70% ethanol (EtOH) until paraffin embedding. They were then embedded in paraffin and tissue slides prepared using methods previously described^[6].

Immunohistochemistry

The tissue sections were deparaffinized by sequential immersion in Tissue-Clear (Sakura Finetek, Tokyo, Japan) for 2 min \times 10 min, 99% isopropanol for 2 min \times 5 min, 95% isopropanol for 1 min \times 5 min, 70% isopropanol for 1 min \times 5 min, and distilled water for 1 min \times 5 min, after which they were gently dried.

The tissue sections were rinsed twice with PBS Ca²⁺/Mg²⁺ for 5 min each time, and then treated with Proteinase K (Sigma-Aldrich, St. Louis, MS, United States) for 10 min, followed by rinsing with PBS. Next, a 5% mixture of normal goat serum (Vector Laboratories, Inc., Burlingame, CA, United States) in PBS was applied for 30 min, after which the primary antibodies were applied overnight in a refrigerator.

The tissue sections were rinsed by applying PBS for 2 min \times 5 min. The secondary antibodies used were CK3 [mouse monoclonal antibody, AE5 (ab77869); Abcam, Cambridge, United Kingdom], a marker for corneal epithelial cells (Abcam); paired box 6 (PAX-6) [mouse monoclonal antibody, AD2.38 (ab78545), Abcam], a marker for a transcription factor important in the development of the eye; octamer-binding transcription factor 3/4 (OCT-3/4) [mouse monoclonal antibody, C-10 (sc-5279), Santa Cruz Biotechnology, Inc., Santa Cruz, CA, United States], a marker for embryonic stem cell activity; sodium potassium adenosine triphosphatase (NaKATPase) [rabbit monoclonal antibody (ab58475), Abcam], a marker for NaKATPase pump function; and Zona Occludens protein 1 (ZO-1) [Guinea pig polyclonal antibody (NBP1-49669), Novus Biologicals, Littleton, CO, United States], a marker for tight junctions.

Slides were washed in PBS Ca²⁺/Mg²⁺ and incubated with a secondary antibody (Alexa Fluor® goat anti-mouse or anti-rabbit antibody; Invitrogen, Eugene, OR, United States) for 2 h at room temperature and in the dark. The slides were then rinsed once more with PBS Ca²⁺/Mg²⁺ before a small drop of mounting medium for fluorescence with 4',6-diamidino-2-phenylindole (DAPI) (Vectashield, Vector Laboratories) was applied and the tissue sealed with a thin glass slide. The analysis was performed on a Nikon fluorescence microscope equipped with DAPI, tetramethylrhodamine (TRITC) and fluorescein isothiocyanate (FITC) filters (360, 490, and 570 nm).

This study was conducted in accordance with the tenets of the Declaration of Helsinki and with permission from the Ethics Committee of the University of Goth-

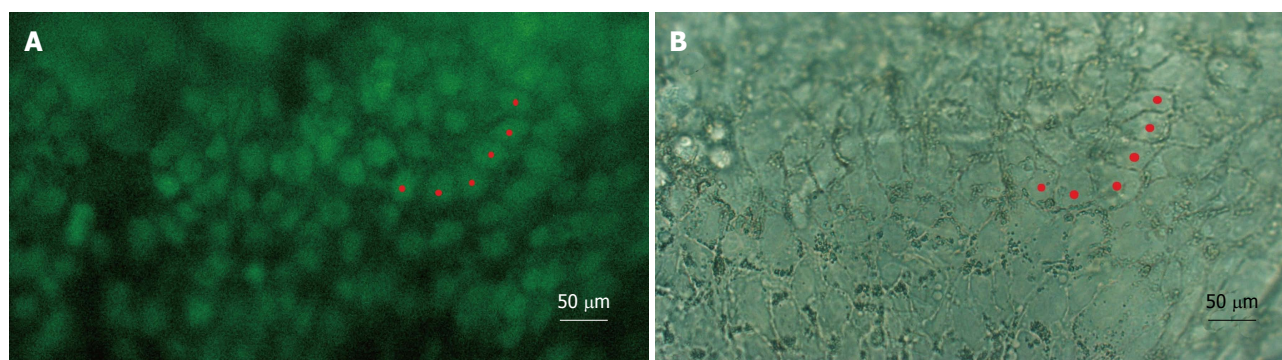


Figure 1 Overview of embryonic stem cells transplanted onto Descemet's membrane. A: Fluorescent cells derived from human embryonic stem cells; B: Outline of the cell walls of the same cells. Red stars mark the same cells in both photos.

enburg.

RESULTS

Morphology of transplanted hESCs

The GFP signal was easily detected in the specimens harbouring stem cells, making it easy to identify the transplanted hESCs. In the tissue sections, it was obvious where the GFP-positive donor cells ended and the recipient cells began.

Several experiments were performed in an attempt to optimize the time required for culturing the hESCs on the donor cornea (data not shown). All of the 18 experiments revealed hESCs attached to the donor cornea. Briefly, the course of events can be described as follows: The transplanted hESCs attached to the DM within 6 h, and cells started to grow in different directions. The cells grew as a monolayer (confirmed later with IHC) and eventually covered the entire surface of the DM.

In all of the experiments where hESCs attached to the DM, they formed clusters of between one and eight cells in thickness. However, as they spread out towards the periphery, they did so in a single cell layer and assumed a flat form as found in intact endothelia. Importantly, the hESCs also lost their roundness and assumed a more polygonal form reminiscent of their intact hexagonal shape (Figure 1). Furthermore, sections of the cells in the single cell layer revealed the flattened morphology typical of endothelial cells (Figure 2). Expression of octamer-binding transcription factor 4 (OCT-4), PAX-6, NaKATPase, CK3 and ZO-1 in transplanted hESCs.

We found expression of octamer-binding transcription factor 4 (OCT-4) in ten out of 18 trials, two with clear signals all over the transplanted area but the other eight showing a very weak signal in 5%-20% of the cells in multilayers. In the remaining eight trials, OCT-4 was completely absent. The hESCs were found to express PAX-6 clearly in nine cases (Figure 2), weakly in five and not at all in four. Out of 18 experiments, 14 were positive for NaKATPase (Figure 2); in two experiments we only found NaKATPase faintly expressed

and in another two not at all. Fifteen out of 18 corneas were negative for CK-3, two had a weak signal and one was deemed positive. Expression of ZO-1 was found in all of the transplanted corneas (Figure 2).

DISCUSSION

We have shown that it is possible to culture hESCs on partially decellularized human corneal DM, where they assume the form of endothelial cells and start expressing relevant proteins.

The sparse OCT-4 expression in the hESCs attached to the DM indicates that the hESCs have started to lose their stem cell qualities and are beginning to adopt features associated with corneal endothelial cells, as shown by the expression of PAX-6, and NaKATPase. The former, PAX-6, is a non-specific indicator of development in the eye. The frequent NaKATPase expression seen in the transplanted hESCs points to high activity of the NaKATPase pump, as would be expected in endothelial cells responsible for actively maintaining the fluid balance within the cornea. We found no correlation between the number of days in pre-culture and expression of the studied proteins. Furthermore, there were no correlations between the expression of OCT-4, PAX-6, NaKATPase, ZO-1 and CK3 and the time during which the cells were grown on DM.

To distinguish between corneal endothelial activity, on the one hand, and epithelial cells, on the other, we used CK3 marker for epithelial cells. As there were some cases that were positive for CK3, we conclude that the differentiation of hESCs towards corneal endothelial cells is not as straightforward as differentiation towards corneal epithelial cells. Hence, there is a need for further optimization of the culture system to ensure differentiation into corneal endothelial cells.

Although there are published studies on culturing of adult endothelial cells (for a review, see Mimura *et al.*^[8]) and on transplantation of hESC-derived, endothelial-like cells onto rabbit corneas^[9], there are no published data on studies of hESCs transplanted onto human corneal endothelial cells.

The knowledge of stem cell culturing (embryonic,

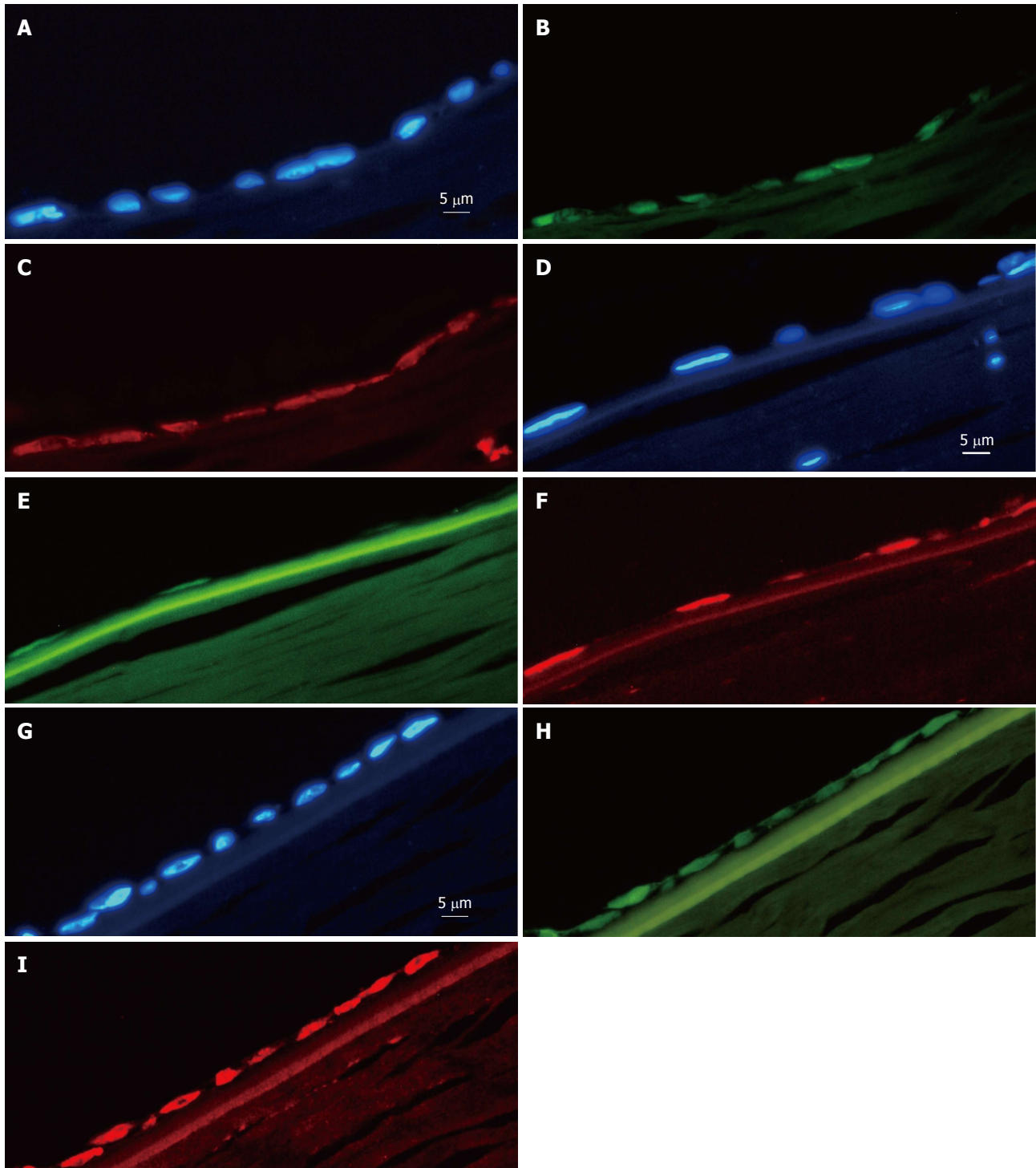


Figure 2 Endothelial parts of three corneal sections: **A-C, D-F, and G-I.** A: Nuclei through a 4',6-diamidino-2-phenylindole (DAPI) filter; B: The same cells through a fluorescein isothiocyanate (FITC) filter [green fluorescent protein (GFP) fluorescence]; C: Expression of the tight junction protein Zona Occludens protein 1 (ZO-1) [tetramethylrhodamine (TRITC) filter]; D: Nuclei seen through a DAPI filter; E: GFP expression (using an FITC filter); F: Expression of sodium potassium ATPase (NaKATPase) (using a TRITC filter); G: Nuclei seen through a DAPI filter; H: GFP expression (FITC filter); I: Expression of the cornea-related protein paired box 6 (PAX-6).

foetal, and adult stem cells) for clinical applications is still rudimentary. Future developments within the field of stem cell research will probably lead to different options for different applications. For the treatment of certain diseases, adult stem cells will be the first choice; but for others, embryonic or foetal stem cells will be more suitable. Stem cell research may in the future give us

a toolbox of specialized cells which can be matched to different clinical and individual conditions. The current study is a step towards better understanding of how embryonic stem cells can contribute to improved function in the human cornea and to finding new treatment strategies for patients with corneal disorders or damage. We have shown that hESCs attach and grow on DM,

and that the cells have the potential to differentiate into corneal endothelial-like cells. However, further optimization of the process is needed. We suspect that the “pre-differentiation” period is key in solving the problem of variation obtained. It seems that just adding ROCK inhibitor is not sufficient for a 100% success rate in differentiation; other, still unknown factors may be used to elevate the efficiency of the differentiation into corneal endothelial cells.

It is possible to get cells originating from hESCs to establish onto a human DM where they grow, and differentiate into corneal endothelial-like cells *in vitro*.

COMMENTS

Background

Serious corneal damage can in many cases only be treated with transplantation. Because of a global shortage of donated human tissue, there is a growing interest in developing stem cell technology as an alternative. Human embryonic stem cells (hESCs) could be an inexhaustible source in corneal damage treatment.

Research frontiers

There are several ongoing studies on culturing adult corneal endothelial cells for transplantation. Some of them seem very promising. However, hESCs would be a complement to these adult cells and have the advantage of being of an earlier origin with the potential for lifelong survival after transplantation.

Innovations and breakthroughs

This is the first report on the interaction between hESCs and the inner parts of the human cornea. When hESCs were transplanted onto the Descemet's membrane (DM) *in vitro* the authors found that the stem cells were able to attach to and grow on the DM in a single cell layer. Furthermore, they changed their morphology from small round cells to flat hexagonal cells. The transplanted stem cells also started to express the proteins paired box 6, Zona Occludens protein 1 and sodium potassium adenosine triphosphatase (NaKATPase) which are also expressed in human corneal endothelial cells.

Applications

These results point to the possibility of using embryonic stem cell-derived

corneal cells for reconstruction of damaged corneas.

Peer-review

This is a well written manuscript evaluating the results of an attempt to get hESCs to differentiate into corneal endothelial like cells *in vitro* on human corneas that had been partially or completely cleared of all existing endothelial cells.

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S- Editor: Ji FF **L- Editor:** A **E- Editor:** Lu YJ



Randomized Controlled Trial

Phase I / II randomized controlled trial of autologous bone marrow-derived mesenchymal stem cell therapy for chronic stroke

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Author contributions: Tsang KS conducted experiments, analyzed data and wrote the manuscript; Ng CPS coordinated the trial and performed statistical analysis of data; Zhu XL and Wong GKC consulted and administered cells and placebos to patients; Lu G reviewed data; Ahuja AT analyzed radio-imaging; Wong KSL and Ng HK contributed intellectual content; Poon WS designed, supervised and monitored the trial, secured grant support, interpreted data and revised the manuscript.

Institutional review board statement: The randomized, controlled, double-blind, phase I / II clinical trial was approved by the Joint Chinese University of Hong Kong - New Territories East Cluster Clinical Research Ethics Committee of Hong Kong Hospital Authority in accordance with the principles of the Declaration of Helsinki and International Conference on Harmonisation - Good Clinical Practice.

Clinical trial registration statement: The clinical trial CREC #2006.425-T was registered at <http://crec.cuhk.edu.hk>.

Informed consent statement: Written informed consent was obtained from all subjects - in the case of vegetative state, from their next of kin.

Conflict-of-interest statement: All authors declared that no conflict of interest exists.

Data sharing statement: No additional data are available.

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Abstract

AIM

To examine the safety and efficacy of mesenchymal stem cell (MSC) therapy for intracerebral haemorrhage with neurological dysfunctions for a year.

METHODS

MSC were *ex vivo* expanded from 29 mL (17-42 mL) autologous bone marrow. Patients were randomized to have two intravenous injections of autologous MSC or placebos in four weeks apart. Neurological functions and clinical outcomes were monitored before treatment and at 12th, 16th, 24th, 36th and 60th week upon completion of the treatment.

RESULTS

A mean of 4.57×10^7 (range: 1.43×10^7 - 8.40×10^7) MSC per infusion was administered accounting to 8.54×10^5 (2.65×10^5 - 1.45×10^6) per kilogram body weight in two occasions. There was neither adverse event at time of administration nor sign of *de novo* tumour development among patients after monitoring for a year post MSC therapy. Neuro-restoration and clinical improvement in terms of modified Barthel index, functional independence measure and extended Glasgow Outcome Scale were evident among patients having MSC therapy compared to patients receiving placebos.

CONCLUSION

Intravenous administration of autologous bone marrow-derived MSC is safe and has the potential of improving neurological functions in chronic stroke patients with severe disability.

Key words: Stroke; Intracerebral haemorrhage; Central nervous system; Mesenchymal stem cells; Cell therapy

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Core tip: Contemporary treatments are ineffective in restoring lost neurological functions after stroke. Many stroke patients were noted to have lesions close to the sub-ventricular zone. The likely beneficial effects of mesenchymal stem cell (MSC) treatment might correlate with the spatial lesion, not part of the sub-ventricular zone where endogenous neurogenesis persists during adulthood, and indirect chaperon effects of MSC promote endogenous neuro-regeneration. We administered MSC intravenously to patients having severe neurological disability and presenting stable baseline scores one year after the onset of intracerebral haemorrhage to eliminate confounding attributes to the observation of MSC-mediated neurological recovery.

Tsang KS, Ng CPS, Zhu XL, Wong GKC, Lu G, Ahuja AT, Wong KSL, Ng HK, Poon WS. Phase I / II randomized controlled trial of autologous bone marrow-derived mesenchymal stem cell therapy for chronic stroke. *World J Stem Cells* 2017; 9(8): 133-143 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v9/i8/133.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v9.i8.133>

INTRODUCTION

Stroke is a common neurological disorder and is a

leading cause of death. More than six million cases of stroke are reported in the world annually^[1]. Approximately 50% of the patients died and most of the survivors are left with various degree of neurological dysfunction. There is no effective treatment for restoring the neurological function of the patients to date. Recent studies in animal models of intra-cerebral haemorrhage demonstrated active neurogenesis in the sub-ventricular zone leading to new neurons of phenotypes of their dead counterparts^[2,3]. Nevertheless, a majority of newly formed neurons die during the early weeks after stroke, and successful replacement only accounts for a small portion of the mature dead neurons^[4]. The feasibility of using a variety of cell types including neural stem cells, embryonic stem cells, umbilical cord blood cells and mesenchymal stem cells (MSCs) to enhance re-innervation has been demonstrated in animal models^[5-8]. The breakthrough opens cellular therapy for stroke. In clinical setting a reliable and accessible cell source is requisite. Bone marrow-derived MSCs, which were noted to generate trophic factors, growth stimulants, signalling regulators and cytokines, might help promote neuro-regeneration and neuro-restoration after stroke *via* neurogenesis, angiogenesis and synaptogenesis^[9]. Cellular therapy employing large numbers of *ex-vivo* expanded viable MSC might be a potential treatment modality to patients after stroke.

An earlier study demonstrated the feasibility and safety of infusion of autologous MSC in patients nine weeks after stroke onset^[10]. In the present study we conducted a phase I / II randomized controlled trial of autologous bone marrow-derived MSC therapy in patients one year after onset of stroke with the aim to study the long-term safety and functional efficacy of intravenous administration of MSC.

MATERIALS AND METHODS

Study design

This study is a randomized, controlled, double-blind, phase I / II clinical trial (CREC #2006.425-T) and was approved by the Joint Chinese University of Hong Kong - New Territories East Cluster Clinical Research Ethics Committee of Hong Kong Hospital Authority in accordance with the principles of the Declaration of Helsinki and International Conference on Harmonisation - Good Clinical Practice. Inclusion criteria are that patient had the onset of stroke for one year ago with stable National Institutes of Health Stroke Scale scores ≥ 7 and Glasgow Outcome Scale score of severe disability and vegetative state at one year after onset of stroke^[11,12]. Exclusion criteria are lacunar syndrome, malignant diseases, severe co-morbidity, hepatic/renal dysfunction and unwillingness to participate. Patients who presented stable baseline scores indicating severe neurological disability were recruited to the study. Informed consent was obtained from all subjects - in the case of vegetative state, from their next of kin. Eligible patients were randomly assigned to the

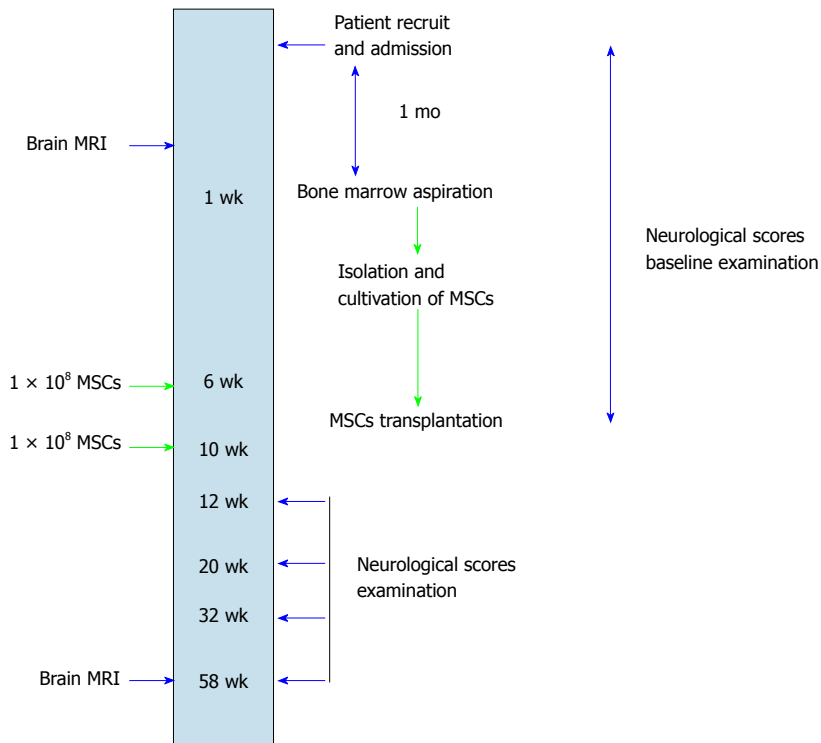


Figure 1 Workup schema for patients recruited to the clinical trial of autologous mesenchymal stem cell therapy. MSC: Mesenchymal stem cell; MRI: Magnetic resonance imaging.

treatment group and control group for autologous MSC therapy (Figure 1). The study protocol was developed according to the guidelines of Consolidated Standards of Reporting Trials available on-line at <http://www.consort-statement.org/>.

Radio-imaging

Computed tomography (CT) scan was conducted at onset of intracerebral haemorrhage. Haematoma volume in mL was computed by using the formula: $\frac{1}{2} \times \text{maximal height (cm)} \times \text{width (cm)} \times \text{anterior-posterior diameter (cm)}$. Magnetic resonance imaging (MRI, 1.5 Tesla) of the brain were performed on the day before the first injection of either MSC or placebos. A follow-up procedure was conducted on patients at 60th week upon completion of the study (Figure 1).

Ex vivo expansion and infusion of MSC

The procedure of MSC expansion described by Le Blanc *et al.*^[13] was adopted with minor modifications. In brief, bone marrow aspirates from the superior iliac crest of patients under local anaesthesia were anticoagulated in 10 IU/mL preservative-free heparin (DBL, Hospira, Melbourne, Australia). Mononuclear cells were enriched by using density-gradient centrifugation in ficoll-hypaque with specific gravity of 1.077 g/mL (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and cultured in low-glucose Dulbecco modified eagles' medium (Invitrogen, Life Technologies, Carlsbad, CA, United States) supplemented with 10% foetal bovine serum (Invitrogen). MSC cultures of approximately 80% in confluence were passaged using 0.05% trypsin (Invitrogen) and *ex vivo* expanded by subcultures in 175 cm² flasks.

On the day of infusion, MSC cultures were enzymatically segregated and dislodged from culture flasks by trypsin digestion, washed with phosphate-buffered saline, sieved to remove cell aggregates *via* 40- μ m filter and re-suspended in 10 mL 5% normal human albumin (Hong Kong Red Cross Blood Transfusion Service) for intravenous injection in five to ten minutes. Another booster bolus of autologous MSC was prepared and administered to patient four weeks thereafter. A placebo of an equal volume of 5% normal human albumin was administered to patients being allotted to the control group. Cultures and cell processing were conducted under conditions meeting the requirements of good manufacturing practices.

Characterisation of MSC

Immunophenotyping of MSC by flow cytometry was reported elsewhere^[14]. Unless stated otherwise, fluorescence-conjugated monoclonal antibodies from Beckman Coulter were used. They were IgG1-FITC, IgG1-PE, HLA-DR-FITC, CD45-FITC, CD3-FITC, CD19-PE, CD16-FITC, CD33-FITC, CD38-FITC, CD34-PE, CD133-PE (Miltenyi Biotec GmbH, Germany), CD29-PE, CD44-FITC, CD73-FITC, CD90-PE, CD105-PE (Serotec, United Kingdom) and CD166-PE were used. At least 10000 events were acquired and signals were analysed by using the Coulter Epic XL MCL flow cytometer (Coulter, Miami, FL, United States).

Procedural details of immunofluorescence staining were described previously^[15]. IgM anti-stage-specific embryonic antigen-4 (SSEA-4, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, United States), IgG_{2b} anti-octamer-binding transcription factor-4 (Oct-4; 1:100, Santa Cruz Biotechnology), IgG₁ anti-Nestin (1:400;

BD Biosciences, San Francisco, CA, United States) were employed.

Cell viability was evaluated by using trypan blue dye exclusion test. Sterility check against microbial contamination was conducted at each MSC passage.

Neurological functional assessments

Patient safety and efficacy of cell therapy were evaluated during the first and second MSC infusion and thereafter at 16th, 24th, 36th and 60th week of the study (Figure 1). The safety of intravenous infusion of either MSC or placebos was assessed in terms of the development of immediate or delayed adverse reactions. Immediate reactions included allergic responses (tachycardia, fever, skin eruption and leucocytosis), local complications (hematoma, local infection at the injection site), vascular obstructions (tachypnea, oliguria, peripheral vascular insufficiency, recurrence of stroke), and systemic complications (systemic infections, increased aspartate aminotransferase, alanine aminotransferase and/or blood urea nitrogen/creatinine levels). Presence of delayed adverse reaction of tumour formation was evaluated by physical examination of skin and oral mucosa and followed up with magnetic resonance imaging (MRI), if necessary. Modified Barthel Index and Functional Independence Measure were monitored by a neurologist being blinded to group allocation and radiological data^[16,17]. Scores of Extended Glasgow Outcome Scale were also used to track the progress of disability of patients over time^[12]. Stroke scale scores, vascular risk factors, medical history and demographic details were recorded.

Statistical analysis

Means, ranges and standard deviations of continuous variables of years of age, kilogram in body weight, milliliter of hematoma and bone marrow, percentages of cell counts and viability were calculated. Assuming that data were normally distributed, non-parametric Wilcoxon's rank sum test was used to compare variables derived from the treatment and control groups in the study. Paired *t*-test with one-sided testing was used to analyse scores of modified Barthel Index, Functional Independence Measure and Extended Glasgow Outcome Scale of patients at time of assessments. Fisher's exact test was applied to examine the incidence of clinical neurological improvement between the treatment and control groups of patients. Differences between groups were regarded as significant if $P \leq 0.05$.

RESULTS

Patient characteristics

We conducted a double-blind, randomized, controlled phase I/II trial to examine the safety and efficacy of autologous MSC therapy in a small cohort of nine patients (four females and five males) with a mean age of 52 years (range: 41-59 years) who had undergone intracerebral haemorrhage (ICH) for a year. CT scan at

time of the onset demonstrated cerebral haematoma of 52 mL (12-75 mL) located in the basal ganglion region of the brains of the nine patients in the study cohort. The sizes of the lesion areas were comparable between the treatment and control groups.

MSC were *ex vivo* expanded from a mean volume of 29 mL (17-42 mL) autologous bone marrow. Patients were randomized to have two intravenous injections of autologous MSC (treatment group of MSC: $n = 5$ or control group of placebos: $n = 4$) four weeks apart. The body weight of patients in the treatment group were statistically lighter than those of the control group [treatment vs control; 54.2 kg (42-60 kg) vs 67.2 kg (64-72.7 kg), $P = 0.03$], however the years of age of both groups were comparable [treatment vs control; 53.4 (48-56) vs 51.5 (41-59); $P = 0.64$]. There was no difference between the severities of disability in terms of neurologic scores of patients assigned to both groups (data not shown).

MSC autograft

Ex vivo expanded MSC at a mean of 4 passages (1-8) were used for infusion. MSC up to passage-8 displayed longitudinal, bi-polar, spindle-shaped and fibroblast-like morphology. Immunofluorescence staining demonstrated expressions of embryonic stem cell marker SSEA-4, transcription factor Oct-4 and neural stem cell marker Nestin; suggesting the pluripotency and neurogenesis of MSC (Figure 2). Flow cytometry demonstrated that they were immunophenotypically positive for CD29, CD44, CD73, CD90, CD105 and CD166 (Figure 3A), but negative for haematopoietic stem cell markers (CD34 and CD133), myeloid progenitor cell markers (CD33 and CD38), leucocyte markers (HLA-DR and CD45), T-cell marker CD3, NK cell marker CD16 and B-cell marker CD19 (Figure 3B).

MSC infusions

Table 1 shows numbers and doses of MSC in 11 episodes of infusion into five patients (three females and two males). A mean of 4.57×10^7 (1.43×10^7 - 8.40×10^7) MSC per infusion was administered accounting to 8.54×10^5 (2.65×10^5 - 1.45×10^6) per kilogram body weight in two occasions except Patient NSCT02 underwent three infusions. Infused cells were immunophenotypically homogenous; HLA-DR-, CD45-, CD3-, CD19-, CD16-, CD33-, CD38-, CD34- and CD133-positive cells were less than 1% on average, whereas CD29-, CD44-, CD73-, CD90-, CD105- and CD166-positive cells were more than 96% (Supplementary Tables 1 and 2). Cell viability was 94.4% (88.5%-99.0%). There was no microbial growth as evident by aerobic and anaerobic cultures of 11 infusates. The control group of four patients (one female and three males) received placebos in an identical manner. No adverse reaction of acute infusion-related toxicity, transient fever, complication in organs or infection was experienced by both groups of patients at time of and a day following MSC administration. There was no sign of tumour development among patients in

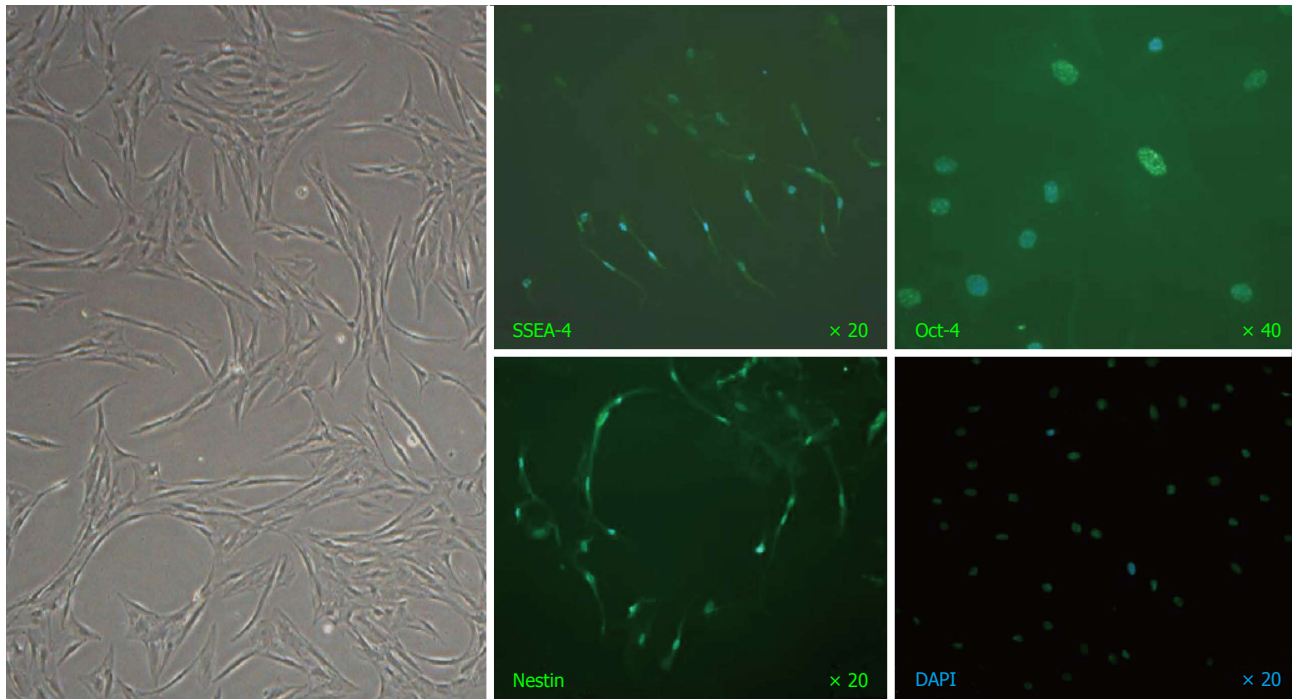


Figure 2 A representative image of mesenchymal stem cells at passage-8 captured under phase-contrast microscopy (left panel) and immunofluorescence staining of stage-specific embryonic antigen SSEA-4, transcription factor Oct-4 and neural stem cell marker Nestin (green fluorescence) with nuclei counterstained by DAPI (blue fluorescence).

Table 1 Characteristics of intracerebral haemorrhage patients and infuses for autologous bone marrow-derived mesenchymal stem cell therapy

Patients	Sex/age in years	Location of haemorrhage	CT readout (cm)	Hemorrhage volume (mL)	MSC passage numbers	Viability (%)	MSC ($\times 10^7$)	MSC/kg ($\times 10^5$)
UPN02	F/50	Basal ganglia, left	4.4 \times 5.8 \times 5.9	75	4, 7	96.8	3.1	5.44
					3-5	92.1	3.2	5.61
					3-6	92.6	3.1	5.44
UPN08	F/48	Basal ganglia, left	4.0 \times 2.0 \times 3.0	12	3, 4	95.8	1.43	2.65
					3, 4	96.5	1.43	2.65
UPN09	M/56	Basal ganglia, right	6.5 \times 5.0 \times 4.0	65	3-8	98.5	7.6	13.1
					2-6	96.8	4	6.84
UPN10	F/55	Frontal lobe, right	5.6 \times 4.5 \times 5.0	63	1-6	88.5	5.5	13.1
					1-6	98	5.6	12.4
UPN11	M/55	Basal ganglia, left	2.4 \times 5.0 \times 3.5	21	1-6	90.5	5.4	9
					1-7	99	8.4	14.5
UPN05	F/59	Basal ganglia, left	6.0 \times 4.0 \times 4.0	48				
UPN06	M/56	Basal ganglia, frontoparietal temporal lobe, right	7.3 \times 3.5 \times 5.0	64				
UPN07	M/41	Basal ganglia, right	4.3 \times 6.4 \times 5.0	69				
UPN12	M/50	Basal ganglia, putaminal, right	NA	55				

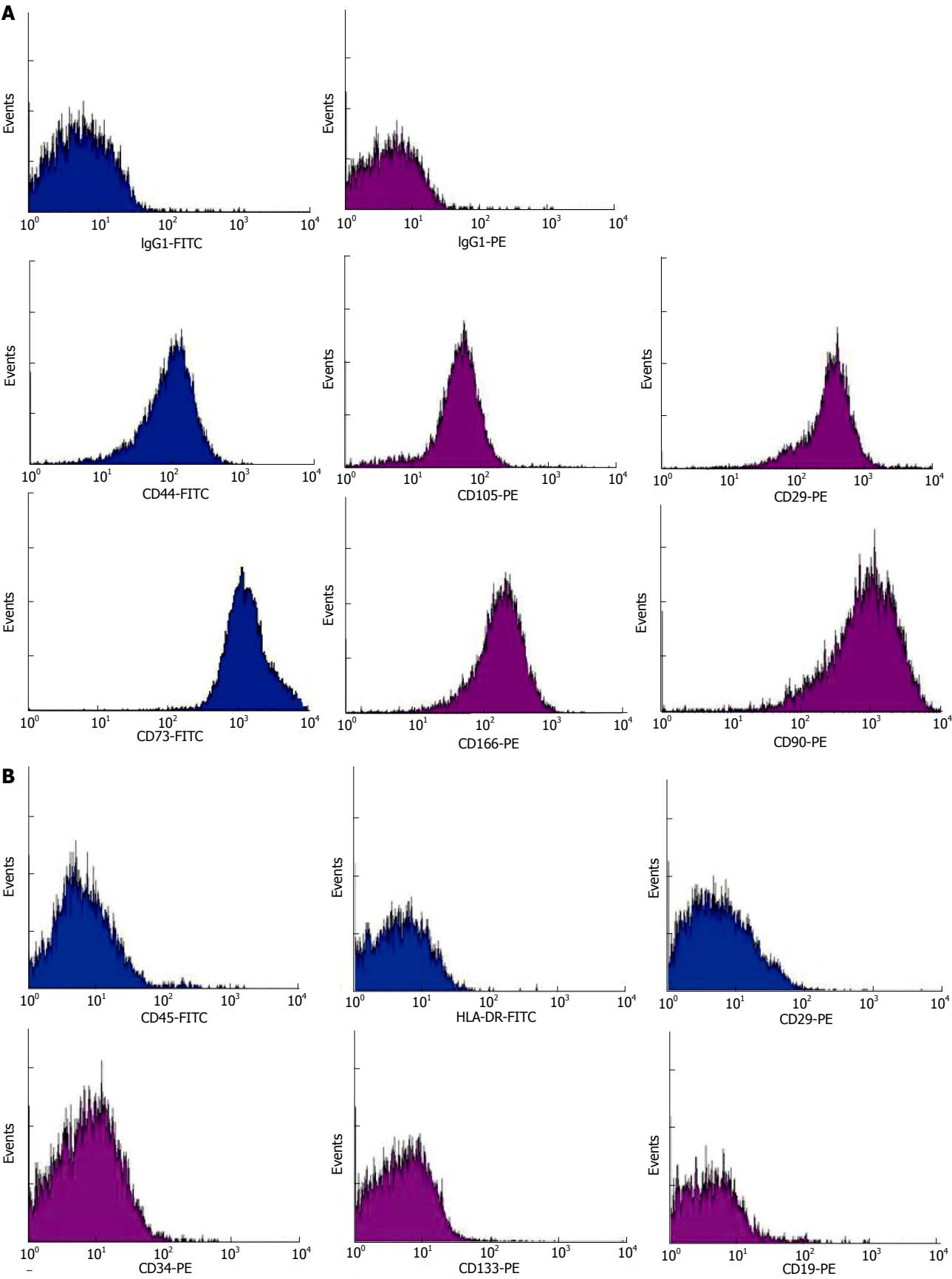
MSC: Mesenchymal stem cell; CT: Computed tomography; NA: Not available.

the study cohort having monitored for a year.

Functional outcomes

Neurological functions and clinical outcomes were monitored before and at 12th, 16th, 24th, 36th and 60th week upon completion of the treatment. In terms of the scores of modified Barthel Index and Functional Independence Measure, the magnitudes of physical and cognitive disability were comparable between the treatment and control groups. Improvements of motor

disability and cognitive impairment were observed over the course of a year among patients undergoing MSC therapy (Tables 2 and 3). Similar progresses were not apparent in the control group receiving placebos (Supplementary Tables 3 and 5). Scores of Extended Glasgow Outcome Scale demonstrated a trend of improvement of clinical outcomes of patients at 24th, 36th and 60th week upon completion of the MSC therapy (Table 4). Evident clinical improvement in patients of both groups were comparable (Patients with higher



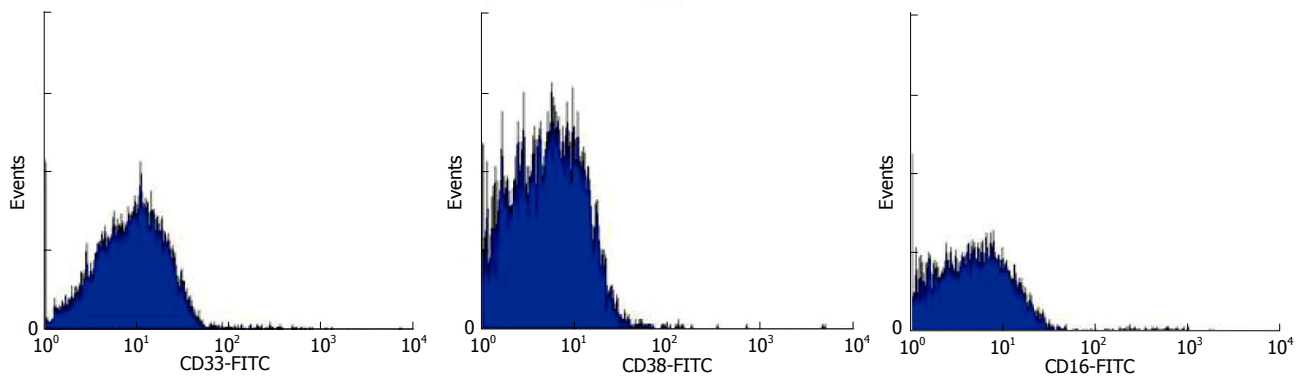


Figure 3 Representative histograms derived from infusates by flow cytometric analyses of mesenchymal stem cell markers CD29, CD44, CD73, CD90, CD105 and CD166 (A) and haemic markers HLA-DR, CD45, CD3, CD19, CD16, CD33, CD38, CD34 and CD133 (B). FITC conjugation in blue and PE conjugation in purple.

Table 2 Modified Barthel indices of the treatment group ($n = 5$) having mesenchymal stem cell therapy at the 1st, 12th, 16th, 24th, 36th and 60th week upon completion of the study

Study duration (wk)	Modified barthel indices					<i>P</i> value
	UPN02	UPN08	UPN09	UPN10	UPN11	
1 st	19	66	0	4	69	0.12
12 th	19	73	0	5	70	
16 th	30	76	0	5	77	
24 th	32	76	0	5	77	
36 th	32	78	1	6	77	
60 th	32	78	1	-	77	0.03

Data derived from different time points were compared to the baseline values established at the first week of the study.

Table 3 Functional independence measure of the treatment group ($n = 5$) having mesenchymal stem cell therapy at the 1st, 12th, 16th, 24th, 36th and 60th week upon completion of the study

Study duration (wk)	Scores of functional independence measure					<i>P</i> value
	UPN02	UPN08	UPN09	UPN10	UPN11	
1 st	36	72	20	83	70	0.07
12 th	35	73	21	88	74	
16 th	40	79	21	89	80	
24 th	41	85	21	89	84	
36 th	44	87	22	102	84	
60 th	42	88	22	-	85	0.03

Data derived from different time points were compared to the baseline values established at the first week of the study.

scores of Extended Glasgow Outcome Scale: MSC vs placebos; 3/5 vs 1/4; $P = 0.52$). There was no re-occurrence of ICH among patients in the study.

Radio-imaging

Comparing MRI brain before MSC injection and at completion of the study, there was no interval change in morphology.

DISCUSSION

In the study we demonstrated the safety, feasibility and

improvement of neurological outcomes of intravenous administration of autologous bone marrow-derived MSC in a small cohort of nine chronic stroke patients one year after intracerebral haemorrhage through a randomized controlled double-blinded phase I / II clinical trial.

Despite advances in neurosurgery and contemporary medical regimes, survivors of intracerebral haemorrhage often suffer long-term to permanent severe disabilities in terms of cognitive impairment and motor dysfunction. Neurological restoration remains poor. There is an imperative to develop therapeutic

Table 4 Scores of extended Glasgow Outcome Scale of the treatment group ($n = 5$) having mesenchymal stem cell therapy at the 1st, 12th, 16th, 24th, 36th and 60th week upon completion of the study

Study duration (wk)	Scores of extended glasgow outcome scale					P value
	UPN02	UPN08	UPN09	UPN10	UPN11	
1 st	3	4	3	3	4	
12 th	3	4	3	3	4	1
16 th	3	4	3	3	4	1
24 th	3	4	3	3	5	0.19
36 th	3	5	3	3	5	0.09
60 th	4	6	3	-	5	0.05

Data derived from different time points were compared to the baseline values established at the first week of the study.

modalities to promote neurological recovery. Stem cell-based therapy has drawn a lot of attention recently and the therapeutic efficacies of various cell types were studied^[18]. Some cell types are deemed difficult for a wide application. Human neural stem cells may be the prototype, however they are not easily harvestable for transplantation unless collected from aborted fetuses or during necropsy^[19,20]. Embryonic stem cells are capable to give rise to all cell lineages, but the application to brain therapy is hindered by the risk of teratoma development and not to mention the ethical controversy^[21]. Therapeutic potentials of human stem and progenitor cells from other sources; including bone marrow mononuclear cells, umbilical cord blood CD34⁺ cells, dental pulp stem cells, adipose-derived stem cells and bone marrow-derived MSC, have also been widely investigated^[22-26]. Previous studies indicated that only a limited number of extraneous cells had eventually implanted and integrated into neural networks of recipients. The numbers of successfully engrafted cells were far less than those lost and died to facilitate neuro-restoration. Nonetheless, the implanted cells elicited the neurological recovery *via* indirect chaperon mechanisms of paracrine signalling of cytokines, chemokines, growth factors, trophic factors, signalling regulators and immuno-modulators, which ultimately stimulated endogenous neurogenesis, angiogenesis and synaptogenesis. It is essential to investigate MSC transplantation as a cell therapy for stroke.

Shortly after the first report on the clinical trial of MSC therapy for stroke^[10], many issues arose to be resolved before MSC therapy can be safely and effectively administered to stroke patients. A plethora of clinical trials of MSC therapy for stroke, including the present study, were conducted in small cohorts of patients rendering the statistical power of safety and efficacy less valid^[10,27-30]. In parallel with studies in large patient cohorts, clinical trials in small cohorts of patients would be more easily manageable and feasible to provide data for meta-analysis. The study serves the goal.

Autologous serum and platelet lysate were used to replace foetal calf serum in the supplement of

basal culture media for MSC propagation in fear of the likelihood of zoonosis^[28,31]. Likewise, animal serum-free chemically modified culture media are feasible alternatives to override the likely hurdle^[27]. There were concerns of loss of stemness, change of functions, senescence and transformation of prolonged cultures of human MSC, nonetheless little report on the clinical trial of human MSC at high passages is available. Honmou *et al.*^[28] reported no side effect on the administration of autologous MSC at passages ≤ 3 in stroke patients during one year of follow-up. More pronounced neurogenesis was observed in a rat stroke model receiving human MSC at earlier passage 2 than counterparts having human MSC at later passage 6^[32]. Bernardo and co-workers reported that long-term *in vitro* cultures of human bone marrow-derived MSC up to passage 25 are not susceptible to malignant transformation^[33]. In the study we investigated MSC at passages up to 8. There was neither morphological changes, phenotypic alterations nor growth senescence. No infusion-related toxicity and complications were experienced by patients at time and upon completion of MSC infusion. Data of the study suggest that MSC up to passage 8 are applicable to clinical use without a compromise of safety over an observation period of a year.

It is intuitive that MSC should rest precisely in the locality of interest in the brain in order to achieve the optimal therapeutic effects. Data of clinical trials of MSC therapy demonstrate that intravenous administration is a feasible approach^[10,27,28,30]. However, the homing of MSC into the brain was shown to be limited and many cells were trapped into the peripheral organs especially the lungs. Alternate means of intra-arterial delivery and intracranial injection using stereotactic device were also reported to be safe and feasible in human^[29,34,35]. Nonetheless, intra-arterial administration was found not superior to intravenous delivery of bone marrow mononuclear cells in a rat stroke model^[36]. Both modes of cell delivery achieve comparable structural and functional outcomes in stroke animals after stem cell therapy despite the low homing efficiency.

The optimal dose of MSC applied to human is largely

unknown. The empirical cell numbers of $0.5 - 5 \times 10^8$ in human are extrapolated from the effective dose of $0.1 - 3 \times 10^6$ cells per rat in rat stroke model^[10,28,37]. Cells of 5×10^7 were administered twice in the first report on the clinical trial of MSC therapy for stroke and better outcome in Barthel index was noted one year post-treatment^[10]. Bhasin *et al.*^[27] transplanted a mean of $5-6 \times 10^7$ MSC and reported neural plasticity. Honmou *et al.*^[28] administered intravenously $0.6-1.6 \times 10^8$ cells per patient and observed reduction of lesion size by $> 20\%$ after one week. In the study a mean of 4.6×10^7 MSC was administered twice and improvements of motor disability and cognitive impairment were noted.

At present, available reports on clinical trials of MSC therapy suggest neuro-restoration, increase of neural plasticity and reduction of lesion volume^[10,27-30,38]. Many stroke patients were noted to have lesions close to the sub-ventricular zone^[39]. The likely beneficial effects of MSC treatment might correlate with the spatial lesion not part of the sub-ventricular zone where endogenous neurogenesis persists during adulthood and indirect chaperon effects of MSC promote endogenous neuro-regeneration^[40]. In the study we administered MSC to patients having severe neurological disability and presenting stable baseline scores one year after the onset of intracerebral haemorrhage to eliminate confounding attributes to the observation of MSC-mediated neurological recovery.

Data of the study suggest that intravenous administration of autologous bone marrow-derived MSC is safe and facilitate the recovery of neurological functions in patients with severe disability long after the onset of intracerebral haemorrhage. Clinical neurological improvement of patients having MSC therapy was evident compared to patients receiving placebos. MSC therapy is effective independently of other treatment courses. The work may help define criteria for future phase III studies.

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COMMENTS

Background

This study was initiated from the randomised Phase II from the Korean Neurologist, suggesting efficacy. Collaborating with the authors Bone Marrow Transplant Unit, the authors were able to be using autologous bone marrow mesenchymal stem cells (MSCs) of sufficient number for two interval infusion.

Research frontiers

To generate efficacy data post-stroke is important. At present there has been no

effective treatment for improving neurological deficits after any stroke illnesses.

Innovations and breakthroughs

There were more data on ischaemic stroke. For brain haemorrhage, clinical data were scarce. This study showed a trend towards improvement for intracerebral haemorrhage.

Applications

The report of this work demonstrated a trend of clinical improvement on two intravenous infusions of autologous bone marrow MSC in four weeks apart.

Terminology

The work provided supportive evidence for phase III randomized controlled trial in the future.

Peer-review

This manuscript is worth publishing, reporting the result of the phase I / II clinical trial of autologous BM-MSC transplantation therapy in a small cohort of patients with chronic brain haemorrhage.

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Stem cell therapy for nerve injury

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Abstract

Peripheral nerve injury has remained a substantial clinical complication with no satisfactory treatment options. Despite the great development in the field of

microsurgery, some severe types of neural injuries cannot be treated without causing tension to the injured nerve. Thus current studies have focused on the new approaches for the treatment of peripheral nerve injuries. Stem cells with the ability to differentiate into a variety of cell types have brought a new perspective to this matter. In this review, we will discuss the use of three main sources of mesenchymal stem cells in the treatment of peripheral nerve injuries.

Key words: Cell-based therapies; Peripheral nerve injury; Stem cells; Mesenchymal stem cells; Bone marrow mesenchymal stem cells; Adipose-derived mesenchymal stem cells; Umbilical cord mesenchymal stem cells

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Core tip: Mesenchymal stem cells (MSCs) can differentiate into many kinds of cell types including Schwann cells (SCs). Since there are limitations for the use of SCs in nerve injuries, it is necessary to know about substitute cell types. So far different sources of MSCs such as embryonic stem cells, bone marrow MSCs, adipose-derived stem cells, etc. have been studied and the existence of beneficial effects on nerve regeneration after injury has been confirmed. Here in this paper, we have collected the latest updates on the use of MSCs from different sources in peripheral nerve regeneration.

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INTRODUCTION

Cell-based therapy in Peripheral nerve injuries (PNIs) has become an important intercession which amends clinical outcome. Contrary to central nervous system, the peripheral nervous system has the potential for

regeneration to a certain extent^[1]. Nevertheless, complete functional recovery is strongly dependent upon the severity of the injury, anatomical site of injury, and the delay before any kind of applied intervention^[2].

What is PNI?

Any harm to the peripheral nerves interrupting their function would be classified as a PNI. In the case of PNI, the connection between the involved nerve fiber and the distal organ would be negatively affected and sometimes even lost, so the distal organ undergoes atrophy due to denervation. In 1%-3% of patients with a traumatic accident, a PNI will almost always be involved^[3,4]. It has been recognized in children suffering falls^[5,6], as a consequence of medical procedures such as surgeries, chemotherapy, radiation^[7-9] and sometimes it has been brought about some chronic conditions like diabetes and cancers^[10,11]. It can also occur as an iatrogenic injury^[12]. There are three main types of a condition causing PNI: Transection, tension, and compression^[13,14]. First of which is commonly caused by penetrating trauma, the second one occurs when a nerve is over-stretched and the third can be reversed if the condition caused the injury is stopped within 8 h. In this article we have mainly focused on transection injuries.

What happens in cellular and molecular level?

A series of cellular and molecular events take place in response to nerve injury. In severe transection injuries (grade V in Sunderland classification or neurotmesis in Seddon classification^[15,16]) caused by penetrating trauma, proximal and distal stumps of the injured nerve undergo pathological changes. "Wallerian degeneration" will occur in distal stump in which injured axons will turn into granule-like debris that will be later cleaned by macrophages^[17]. Proximal stump also firstly retracts back to node of Ranvier^[18] and then tries to reach the distal stump by giving rise to outgrowing axons^[19,20] while activated Schwann cells (SCs) transform into regenerating phenotype and proliferate in the distal stump to form longitudinal columns called "bands of Büngner" which are essential to guide the outgrowing axons^[21]. However, mentioned events along with the secretion of neurotrophic factors by SCs make a great environment for axonal stumps to meet, but the slow rate of axon regeneration which is location-dependent but is usually stated as 1 mm/d^[22], almost always fails these processes and leads to impotency of activated SCs^[23], misguidance of outgrowing axons and target organ atrophy due to prolonged lack of innervation^[24].

Therapeutic strategies

In such cases, the Gold-Standard therapeutic strategy is to join the proximal and distal stumps of the damaged nerve through surgical interventions. Yet, when the gap is too wide to be repaired without stretching the nerve fiber, a nerve graft or a conduit is needed to bridge

the gap. Although nerve grafting is the gold standard technique^[20,25], this often leads to consequences such as donor site unwholesomeness for autologous grafts and graft rejection for heterologous grafts. On the other hand, conduits provide a guiding channel for axonal outgrowth and they can also serve as a vehicle to deliver essential growth factors and supporting cells^[20,26-29]. In recent years, cell transplantation has been proposed as a method of improving peripheral nerve regeneration. SCs activated in response to nerve injury, as previously described play a key role in Wallerian degeneration and formation of bands of Büngner. These features make SCs the most suitable supporting cell candidate to transplant, but regarding other important features of SCs such as the difficulty of harvest, the slow expansion in culture and a high immunogenicity^[30,31], SCs could not make the ideal supporting cells. So attentions have moved towards the use of differentiated and undifferentiated types of stem cells which have the capacity to transform into a variety of different cell types in presence of particular factors.

Use of stem cells

Stem cells are undifferentiated cells of an organism being capable of giving rise to indefinitely more cells of the same type, and other types of cells by differentiation. Stem cells commonly come from two main sources: Embryos (embryonic stem cells), which can be harvested during embryonic period and adult tissues (adult stem cells) that are available in all the tissues in the body. Stem cells are classified by their capability to differentiate into other cell types. Unipotent stem cells (like muscle stem cells) can only give rise to cells of their own type. Oligopotent stem cells can differentiate into a few cell types, like myeloid stem cells. Multipotent stem cells have the ability to differentiate into a nearly related type of cells, like hematopoietic stem cells which not only can produce red blood cells but also can give rise to white blood cells and platelets. Pluripotent stem cells can differentiate into almost all cell types and the examples include embryonic stem cells and the cells from ectodermal, mesodermal and endodermal layers. Totipotent stem cells are the only ones which are able to give rise to all possible cell types, the example is the first few cells that result from the division of the zygote and the fertilized zygote itself.

Mesenchymal stem cells

In this review we mainly focused on mesenchymal stem cells (MSCs), the multipotent stem cells which can be obtained from various sources such as bone marrow, umbilical cord and amniotic fluid, adipose tissue, and also teeth. These cells are characterized morphologically by a small cell body containing a round nucleus with a clear appearance and a prominent nucleolus. Cells have a few long cell processes and the cytoplasm contains Golgi apparatus, mitochondria, rough endoplasmic reticulum and ribosomes. They are spread widely in the

extracellular matrix containing a low amount of reticular fiber.

All-together, this paper will discuss the recent progress in the use of cell-based therapies and of interest the use of MSCs for peripheral nerve regeneration. It will summarize the perspectives of employing main sources of MSCs to speed up the healing process in injured peripheral nerves and involved mechanisms.

SURGICAL TECHNIQUES

The most common donor nerve used for autograft is Sural nerve which is a sensory nerve, hence it cannot be the proper choice for the repair of nerves with mixed motor and sensory or motor constituent^[20,32]. Regarding to the complications of nerve autografts, researchers have focused on using substitute options to bridge the wide gaps with no harm to nerve ends. Various absorbable biomaterials have been used to make conduits and authors worldwide reported different results^[20,26-29]. Conduits can be autogenous or synthetic. Autogenous conduits such as vein conduits sometimes accompanied by muscle or platelet-rich plasma components regardless of good outcomes require a donor site for harvesting^[33,34]. A wide range of synthetic conduits made of collagen, polycaprolactone, polyglycolic acid and polyester have also been studied. Taras *et al*^[35] used collagen conduits and reported good sensory nerves recovery. Wangenstein *et al*^[36] and Ashley *et al*^[37] showed that collagen conduits can have beneficial effects in clinical experiments as well as preclinical experiments with using them in trauma patients and infants with brachial plexus injuries respectively. They run a follow-up survey and monitored 5 infants with transplanted collagen conduits and reported significant motor recovery. Lohmeyer *et al*^[38] also used collagen conduits for nerve reconstruction and reported a 55% of two-point discrimination and 77% of protective sensation recovery. Boeckstyns *et al*^[39] used collagen tubules for recovery of the injured median and ulnar nerves and Sosa *et al*^[40] used collagen tubules containing platelet-rich fibrin for a patient with ulnar neuroma and both of them reported significant motor and sensory recovery. Mackinnon *et al*^[18] used polyglycolic acid tubes in 15 patients with 17 mm nerve gaps and found that despite 14% of them having poor recovery, 86% of them showed excellent (33%) and good (55%) signs of recovery. Battiston *et al*^[27] used polyglycolic acid conduits and muscle-vein conduits to see their difference healing properties. Results showed no significant difference between two groups. Weber *et al*^[41] evaluated the beneficial effects of polyglycolic acid tubes compared to neurotaphy and nerve autografts and reported that in gaps of less than 4 mm or more than 8 mm, polyglycolic acids provided better recovery. Despite great improvements in surgical techniques and instruments, this field will have to be more and more investigated to make an optimal combination of cells and neurotrophic factors accompany a conduit to

amend clinical outcomes.

IMPORTANT ROLE OF NEUROTROPHIC FACTORS

For axonal outgrowths are very slow to form and in severe cases it takes a long time for them to reach the distal stump, and on the other hand it is critical for activated SCs to innervate quickly in order to remain in their active form, thus administration of exogenous neurotrophic and growth factor with the ability of speeding up the mentioned processes has gathered attention. Neurotrophic factors are proteins which are necessary for many vital neural activities particularly in the regeneration of neurons after injuries^[42-45]. Brain-derived neurotrophin factor (BDNF) plays a key role after neural injuries and showed to have advantageous effects on outgrowing axons^[46,47]. Nerve growth factor (NGF) have also a beneficial effect on the elongation of outgrowing sensory axons additional to enhancing SCs motility^[48-50]. Glial cell line-derived neurotrophic factor (GDNF) acts like a chemoattractant for SCs^[48-50]. Sox11 is a very important transcription factor upregulating in response to PNI^[51]. Its expression can affect myelination and axonal elongation and levels of BDNF^[52-56]. It also can help with the survival of neurons through the expression of TNF receptor-associated factor-associated NF- κ B activator (TANK)^[51-55]. Vascular endothelial growth factor (VEGF) can improve outcomes of nerve regeneration through improving microcirculation^[57]. Insulin-like growth factor (IGF) found to have stimulant effects on mitosis of SCs and axonal elongation^[58]. Mohammadi *et al*^[59] used silicon tube with hepatocyte growth factor (HGF) filling and reported improved muscle atrophy. Li *et al*^[60] also reported that same beneficial properties of HGF in combination with acellular nerve allograft. Mohammadi *et al*^[61] reported improved recovery after using silicone tube filled with adrenocorticotropin hormone (ACTH). Emel *et al*^[62] have reported that IGF-1 has a better effect on PNI compared to Platelet-rich plasma. Regardless of how much it could be helpful to use the combination of conduits and neurotrophins, it is still important to hold SCs at their active type because over a short period of time they lose their capacity for remaining active, researchers have had invented methods to transplant newly activated SCs to the site of injury or to use cell types which are able to transform into SCs or SC-like cells to support the healing process.

SCs IN NERVE REGENERATION

SCs actively produce cell adhesion molecules, neurotrophins and growth factors and they can also serve as a scaffold allowing axonal sprouts to grow through their basal lamina^[63-66]. They can also produce regulatory factors to help axonal outgrowth^[67,68]. Despite promising results in preclinical experiments, clinical studies did not gain good results because the difficulties with

harvesting^[68,69] and culture of SCs^[70] and the fact that prolong denervated SCs lose the ability to stimulate regeneration^[71].

STEM CELLS USED IN PNIs

Because of stem cells' potentials they have become a source of cells which act as an alternative for SCs in peripheral nerve regeneration^[70,72-74]. Stem cells as previously described, are biological progenitor cells which are undifferentiated and have the ability to produce more undifferentiated stem cells like themselves through mitosis. In addition, they can differentiate into almost all kinds of cell type depending on trophic and tropic factors they are exposed to. In the case of nervous system, stem cells have the ability to differentiate into supporting cells including oligodendrocytes, astrocytes, microglia, SC-like cells, and neurons themselves^[75], so they can be differentiated *in vitro* before transplantation and can also be transplanted in their undifferentiated form allowing to differentiate *in vivo* at the site of injury. An ideal choice of stem cell would be depended on the important features of the cells, like the ease of harvesting through noninvasive procedures, rapid expanding in culture and low immunogenicity^[30,31]. Many kinds of stem cells with different sources have been studied, among them, MSCs having mentioned features, have been suggested as a potential cell line to enhance nerve regeneration. MSCs are multipotent stromal cells which can differentiate into a variety of cell types. Three main sources of MSCs will be discussed in following sections.

Bone marrow mesenchymal stem cells

Several studies have reported that bone marrow mesenchymal stem cells (BMSCs) can be induced to differentiate into mesodermal, ectodermal and endodermal lineage^[76-80]. Interestingly they can differentiate into SC-like cells and ameliorate neural regeneration by releasing neurotrophic and growth factors, BDNF, GDNF, myelin basic protein^[81] and by regulating SCs behavior^[82]. These good effects seem to be irrelevant to their differentiation state because both differentiated and undifferentiated BMSCs represent positive molecular, electrophysiological, histological and behavioral effects in preclinical experiments^[83]. Regarding some problems in harvesting BMSCs like the need of performing invasive and painful procedures that might yield a low number of cells, BMSCs have some disadvantages in clinical studies. Wang *et al.*^[84] compared the combination of BMSC-SCs and Adipose-derived stem cell SCs (ADSC-SCs) with acellular grafts to bridge the sciatic gaps of 15 mm and reported the greater regeneration recovery at the presence of BMSC-SCs and ADSC-SCs. Hu *et al.*^[85] used BMSC seeded grafts for the recovery of 50 mm median nerve injury in monkeys and found that the healing process with good functional and morphological outcomes was close to autografts. Cuevas *et al.*^[86,87] found that using BMSCs have beneficial effects on rat models of PNI with

injured sciatic nerves. They have also run a follow-up experiment to assess the healing process and reported a significant improvement in sciatic nerve-injured rats with transplanted BMSCs compared to control group. Chen *et al.*^[81] used silicon conduits filled with BMSCs and assessed the recovery process measuring the number of growing axons and muscle atrophy along with walking test and reported their beneficial effects on mentioned indices highlighting the role of neurotrophic factors and myelin basic protein upregulation and not the increase in the number of SCs. Haghighat *et al.*^[88] and Mohammadi *et al.*^[89] also showed that using vein conduits with undifferentiated BMSCs can cause a significant increase in the number and diameter of growing axons and functional improvement consequently. Studies showed that differentiated BMSCs can have a better impact when used in combination with acellular nerve allografts rather than undifferentiated BMSCs^[90]. It has been demonstrated that using BMSCs in PNIs can have similar outcomes as in use of autografts. Studies showed that BMSCs can possibly improve the outcome of nerve regeneration by modulating the behavior of SCs along with expressing neurotrophins^[82]. Caddick *et al.*^[79] found that BMSCs can be induced to differentiate into SC-like cells representing SCs markers such as S100, P75, and GFAP. It has been reported that with the use of cytokines, rat BMSCs can be transformed into SC-like cells which were capable of myelinating PC12 cells *in vitro* after 2 wk as well as increasing the myelinated axons in a rat model of PNI after 3 wk^[91]. It has been shown that BMSCs apply their beneficial effects in a dose-dependent manner^[92].

Adipose-derived mesenchymal stem cells

Adipose-derived mesenchymal stem cells (ADSCs) are another source of multipotent stem cells with the ability of transforming into all three germinal layers^[93,94] and additionally has been showed to give much greater numbers of cells compared to other adult tissues^[95], with minimally invasive surgical procedures and a very simple isolation protocol including washing; diffusing with the aid of enzymatic agents; centrifugation and removal of red blood cells (RBCs). This protocol gives a cellular fraction containing various cell types. Among them, ADSCs of interest adhere to the plastic wall of the container and proliferate quickly, so it can be easily recognized and separated from other cells. Studies showed that ADSCs can be induced to express glial cell markers such as S100B, GFAP and P75 neurotrophin receptors *in vitro*^[69]. Also in the case of ADSCs, it has been demonstrated that *in vitro* differentiation into SCs could not bring any further melioration probably because of ADSCs natural capacity of *in vivo* differentiation into SCs^[65]. Di summa *et al.*^[65] demonstrated that ADSC-SCs, as well as BMSC-SCs, can be used for the repair of rat sciatic nerve injury and since unlike the BMSCs, ADSCs can be easily harvested and expanded, they would be a better choice in PNI injuries. Erba *et al.*^[96] transplanted undifferentiated ADSCs in poly-3-hydroxybutyrate conduit to assess the

axonal outgrowth and the transplanted cells capacity to transform at the site of injury. They reported the increase in the number of SCs and regeneration however researchers could not detect any transformation into neither glial nor neural cells. A similar result has been reported by Santiago *et al*^[97] and the possible mechanism suggested by the authors through which the regeneration has been enhanced, was the expression of neurotrophins. Other similar results have been reported by other researchers^[98,99]. Wei *et al*^[100] showed that ADSC filled conduits have the same regenerative effects in rat sciatic nerve injury as SC filled conduit. Researchers found that ADSCs cannot be differentiated into SCs *in vivo* despite *in vitro* differentiation^[101]. It has been demonstrated that undifferentiated ADSCs can release neurotrophins but at a lower extent^[102]. Oliveira *et al*^[103] used polycaprolactone conduits seeded with MSCs and showed the improvement of myelination and function compared with empty conduits. Another research group used collagen conduits with collagen gel containing ADSCs filling and results showed that improvement was similar to nerve autografts^[104].

Umbilical cord mesenchymal stem cells

Regarding ethical concerns with the use of umbilical cord mesenchymal stem cells (UC-MSCs) and limitation of its availability, there is still proofs which show they are superior to other adult stem cell with different sources: First, they can be collected in great numbers without causing any harm to donor simply from discardable tissues after childbirth; second, as they will be collected at the perinatal period, they are less likely to have genetic damages^[105]; third, they are younger than other adult stem cells so they can undergo higher number of mitosis and can be much more expanded in culture^[106]; fourth, while they lack HLA-II, they have much lower immunogenic properties compared to other adult stem cells^[107]. Matsuse *et al*^[108] used tubes filled with SC-like cells which have been previously formed as a result of UC-MSCs differentiation and showed that they can promote axonal regeneration. Same results have been demonstrated by Kuroda *et al*^[109] and Pereira *et al*^[110]. Peng *et al*^[111] demonstrated that SC-like cells can secrete BDNF, Neurotrophin-3, and NGF *in vitro* and when combined with PCI2 cells, axonal growth was seen.

CONCLUSION

To improve peripheral nerve regeneration for better sensory and motor recovery, the use of stem cells and especially MSCs would be greatly helpful. These cells not only can differentiate into SCs *in vitro* but also are able to transform into SCs directly at the site of injury. Furthermore, administration of stem cells, can regulate the activity of native SCs, modify the inhibitory regenerative environment, improve myelination and cell survival and enhance neurotrophic activity. In summary,

MSCs with such suitable properties as the ease of harvesting especially in the case of ADSCs and the lower risk of immunogenic activities have got a great potential to improve the regeneration process. Thus, for sure by further investigations, significant improvements in neural regeneration by the help of MSCs will be obtained.

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Role of aryl hydrocarbon receptor in mesenchymal stromal cell activation: A minireview

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Abstract

Mesenchymal stromal cells (MSCs) possess great therapeutic advantages due to their ability to produce a diverse array of trophic/growth factors related to cytoprotection and immunoregulation. MSC activation *via* specific receptors is a crucial event for these cells to exert their immunosuppressive response. The aryl-hydrocarbon receptor (AhR) is a sensitive molecule for external signals and it is expressed in MSCs and, upon positive activation, may potentially regulate the MSC-associated immunomodulatory function. Consequently, signalling pathways linked to AhR activation can elucidate some of the molecular cascades involved in MSC-mediated immunosuppression. In this minireview, we have noted some important findings concerning MSC regulation *via* AhR, highlighting that its activation is associated with improvement in migration and immunoregulation, as well as an increase in pro-regenerative potential. Thus, AhR-mediated MSC activation can contribute to new perspectives on MSC-based therapies, particularly those directed at immune-associated disorders.

Key words: Mesenchymal stromal cells; Aryl-hydrocarbon receptor; Cell activation and immunosuppression

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Core tip: The aryl-hydrocarbon receptor (AhR) is an endogenous sensor expressed in mesenchymal stromal cells (MSCs), regulating their immunomodulatory function. Therefore, in this review, we summarize important reports that demonstrate that AhR activation can substantially modulate the function of MSCs by mechanisms associated with: (1) The induction of the death signal in pro-inflammatory cells; (2) the suppression of pro-inflammatory genes/cytokines; (3) the improvement of

migration and regenerative potential in acute inflammatory models; (4) the inhibition of mesodermal differentiation; and (5) the up-regulation of global immunosuppression. Thus, the influence of AhR activation on MSC function can establish new perspectives on MSC-based therapies, especially for immune-associated diseases.

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INTRODUCTION

Multipotent mesenchymal stromal cells

Multipotent mesenchymal stromal cells (MSCs), also referred to as mesenchymal stem cells, were originally described by Alexander Friedenstein in 1976 as non-haematopoietic marrow cells in culture^[1]. MSCs were identified as stromal cells that present plastic adherent characteristics and the ability to form *in vitro* fibroblast-like colonies (CFU-F). In 1991, Caplan defined MSCs as a supportive cell population capable of differentiating into several mesodermal cell lineages including muscle, bone marrow stroma, fibroblasts, osteocytes, adipocytes and chondrocytes^[2].

Phenotypically, MSCs are characterized by the expression of surface membrane molecules such as endoglin (CD105), NTSE (CD73), and Thy-1 (CD90) and the lack of expression of haematopoietic (CD45, CD34, CD11b/c and CD19) and endothelial (CD31, KDR) markers and of HLA-DR, an immune-associated molecule linked to major histocompatibility complex class II (MHC II)^[3]. In addition, MSCs resemble vascular pericytes, and due to their wide perivascular distribution^[4,5], these cells can be identified and expanded *ex vivo* from a multitude of tissues and organs, for instance: (1) Bone marrow^[6]; (2) the umbilical cord^[6]; and (3) adipose tissue^[7], highlighting MSCs as a very attractive cell subpopulation for several clinical applications.

From a therapeutic perspective, MSCs possess advantages such as low immunogenicity, migration to injured tissues and the production of various trophic/growth factors (e.g., cytokines, chemokines and diverse growth factors), which may be related primarily to the mechanisms of immunoregulation, anti-fibrosis, the induction of endogenous tissue progenitor cells, anti-apoptosis, pro-angiogenesis and chemoattraction. Moreover, MSCs may act as effector agents in the modulation of internal gene expression by releasing extracellular microvesicles enriched with small regulatory RNAs^[8-10].

In light of their functional multipotentiality, MSCs are essentially distinguished from other cells by retaining immunomodulatory properties that globally reduce the inflammation process, suppressing cellular

alloreactivity. In this regard, studies have shown that the infusion of MSCs reduces local and systemic tissue injury in distinct experimental models, e.g., neural encephalomyelitis^[11], pulmonary fibrosis^[12], kidney injury^[13] and heart inflammation^[14], mainly *via* shifting from a pro-inflammatory to an anti-inflammatory profile. Thus, the immunosuppressive abilities of MSCs may be useful to repair tissue damaged by immune system aggression, for instance: (1) Crohn's disease^[15]; (2) ulcerative colitis^[16]; (3) graft-versus-host disease (GVHD) followed by halogen transplantation^[17]; and (4) organ rejection in transplants^[18]. However, the majority of clinical trials with MSCs remain in phase I / II studies, and most have not clearly described a precise therapeutic response^[19]. In this context, the complete elucidation of the mechanisms associated with the *in vivo* therapeutic effects of MSCs remains a target of intense investigation.

To date, scientists have considered MSCs a heterogeneous population with several factors that can interfere in their therapeutic efficacy, such as phenotype, proliferation, secretory profile, tissue origin, donor age, culture and expansion method conditions (i.e., growth factors, cell confluence, passages, oxygen pressure and biomaterials)^[20,21]. Considering MSCs a manufactured "product" for cell-based therapy, it is essential to standardize operational processes, which must be in accordance with guidelines assigned by the international programme of good manufacturing practices, also known as "GMP". Thus, given the high heterogeneity of cultured MSCs, it is not surprising that MSC-based therapies have not yet become a reality in operating centers distributed in several countries.

In an attempt to establish a global organizational process for MSC therapeutic programmes, there are potential strategies for refining the preparation and application of MSC cultures. According to several described approaches, the activation of MSCs *via* specific receptors is an innovative and accessible methodology for standardizing the use of these cell populations. Studies have found that MSCs express certain key receptors (e.g., TLRs, TNFRs, INFRs) that are activated by the inflammatory microenvironment, modulating its immunosuppressive activity^[22,23]. This phenomenon was already demonstrated *in vitro* and *in vivo*, where important molecules (i.e., TNF- α , INF- γ , PAMPs, DAMPs, IDO, iNOS, PGE-2) and signalling pathways (i.e., PKR, STAT-1, NF- κ B) were shown to be regulated during MSCs activation. In fact, one study found that MSCs exposed to IFN- γ became activated and efficiently suppressed the deleterious effects of an *in vivo* GVHD experimental model almost five-fold more strongly than unstimulated MSCs^[24]. However, the precise role of each receptor, its molecular interactions and its impact on the biology of MSCs yet remain to be investigated.

ARYL-HYDROCARBON RECEPTOR

The aryl-hydrocarbon receptor (AhR) is a member of

the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family of transcription factors and is characterized as ligand-dependent transcriptional regulator acting on the modulation of a distinct number of genes associated with several biological processes including: (1) The cell cycle; (2) apoptosis; (3) hypoxia; (4) the circadian cycle; (5) differentiation; (6) haematopoiesis; (7) migration; and (8) the immune response^[25]. AhR is considered a multifunctional sensor that responds to toxic/pollutant signals from the environment (*e.g.*, dioxins, pollutants and by-products of metabolism), promoting the regulation of gene expression in responsive cells. AhR can be stimulated by a myriad of specific endogenous or exogenous ligands called hydrophobic aromatic hydrocarbons [*e.g.*, polycyclic aromatic hydrocarbons (PAHs), halogenated aromatic hydrocarbons (HAHs) and planar polychlorinated biphenyls (PCBs)], which can be represented by two main classes: (1) Synthetic and non-biological: *e.g.*, dioxins and dibenzofurans; and (2) natural and biological: *e.g.*, carotenoids, flavonoids and tryptophan-derived metabolites, such as kynurenines^[26,27].

AhR activation starts when a chemical signal enters the target cells and binds with strong affinity to the AhR cytosolic multiprotein complex, which is associated with actin filaments in the cytoplasm. This complex is composed of two Hsp90 chaperone molecules, along with co-chaperones such as hepatitis B virus X-associated protein (XAP2 or AIP) and p23 protein. After stimulation, AhR changes its conformational structure to present the nuclear localization sequence, which promotes its own translocation from the cytoplasm to the nucleus *via* the importin β protein. In the nucleus, the AhR-ligand complex detaches from the triplex protein (hsp90/XAP2/p23) to form a dimer with a nuclear protein responsible for AhR translocation, ARNT, which converts AhR to an active isoform with elevated affinity for DNA. Then, the AhR-ligand-ARNT complex binds to a specific promoter regulatory region on DNA [5'-T (N) GCGTG-3'] known as the dioxin-responsive element/sequence (DRE), which is located upstream of the specific *CYP1A1* locus or other genes responsive to the AhR signal. In contrast, the dimerization of ARNT with AhR repressor protein (AhRR) leads to non-association of the AhR-ligand complex and ARNT protein, and consequently, the AhR-ligand complex exposes its nuclear export sequence to the cytoplasm and is further conducted to the ubiquitination and proteasome degradation process (Figure 1)^[28,29].

AhR is closely linked to the regulation and control of immunity, and there is a substantial amount of evidence supporting the hypothesis that AhR may influence PAH/HAH/PPB-mediated immunoregulation^[27,30]. Thus, some reports have shown that AhR activation by particular ligands (*i.e.*, LPS, tetrachlorodibenzo-p-dioxin or TCDD, tryptophan metabolites) can differentially modulate various effects on immunological cells, for example: (1) The function and development of regulatory T cells; (2) the differentiation of Th17 cells; (3) the generation and activity of monocytes and dendritic cells^[31-33]; (4) the

growth and maturation of mast cells; (5) differentiation/maturation and antibody production by B cells; (6) polarization and cytokine production in macrophages^[34,35]; and (7) haematopoietic stem cell expansion, migration, and plasticity^[36,37]. Another emerging aspect associated with AhR transcriptional biology involves its cooperative relationship with other signalling pathways, which may interact with AhR or by antagonism, such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), or by synergism, such as the signal transducer and activator of transcription 1 (STAT-1) and the nuclear factor (erythroid-derived 2)-like-2 factor (Nrf2). These multiple interactions of different signalling pathways can generate distinct responses according to the nature of the stimulus and the cell type target and thus qualifies as a tissue-specific molecular interchange^[26,29,38].

Functionally, AhR can regulate an extensive number of protein-coding genes, specifically those associated with xenobiotic metabolizing enzymes, such as *CYP1A1*, which is a member of the superfamily of oxidative enzymes called cytochrome P-450 monooxygenases^[28]. Among the potential ligands related to AhR activation, the tryptophan degradation products (*i.e.*, tryptamine and kynurenine) are considered natural endogenous stimuli. Under normal conditions, these metabolites are classified as weak inducers, but after a physiological disturbance, their concentration may rise abruptly, leading to strong activation *via* *CYP1A1* signalling^[28]. In this sense, we can assume that an environment of intense inflammation and tissue injury may contain sufficient tryptophan-derived products for MSC activation *via* AhR, improving the MSC-mediated immunotherapeutic responses. According to these findings, we believe that the immunomodulatory potential of MSCs can be strictly regulated by AhR, and their activation may be essential for MSCs to exert their immunosuppressive response. Indeed, some PAH/HAH-derived metabolites themselves can, either directly or indirectly *via* AhR, down-regulate immune-associated pathways such as the antigen-specific T and B cell responses, compromising lymphocyte development. However, the influence of AhR on the regulation of MSC-induced immunosuppression remains poorly investigated^[30].

AhR ACTIVATION IN MSCs

To explore the participation of AhR in MSC activation, it was predicted that MSC priming by AhR is a mechanism intimately associated with its immunotherapeutic response. According to this perspective, it has been shown *in vitro* that MSCs, under standard conditions, support the growth/differentiation of B lymphocytes, but when the MSCs are pre-stimulated by AhR agonist (*i.e.*, DMBA), these cells exert an inverse immunoregulatory response, inducing apoptosis by cell-cell contact in CD43⁺ pro/pre-B cells. This cell death signal is regulated mainly *via* a specific soluble stromal cell-dependent death signal that is presumably regulated by its responsive *AhR* gene, *CYP1A1*^[10,30,39,40]. Later, the authors of the same study

reported that the addition of a precise and competitive inhibitor of AhR, α -naphthoflavone (α -NF), blocked DMBA-induced pre-B cell apoptosis in these bone marrow cell co-cultures^[39].

Subsequently, another work showed that the activation of AhR in MSCs can also modulate their secretory profile. In this report, the MSCs were stimulated with AhR-specific ligands (*i.e.*, DMBA and TCDD), and after stimulation, these cells had their production of mRNA/protein of interleukin-6 (IL-6) suppressed through a process partially regulated by the coactivation of NF- κ B signalling pathways^[41]. IL-6 is required for the growth and terminal differentiation of progenitor blood cells, and its aberrant expression is reportedly associated with autoimmune-related disorders (*i.e.*, systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis)^[42-44]. Thus, this evidence illustrates the intrinsic importance of AhR-mediated MSC activation, highlighting the role of the IL-6/AhR axis in the regulation of the immune system.

Additionally, it was observed that the therapeutic abilities of MSCs can be modulated by AhR activation. The MSCs were activated by AhR-specific agonists (*i.e.*, TCDD and cockroach allergen extract) and showed increased CYP1A1 and CYP1B1 expression. This process was accompanied by an elevated migration potential *in vitro*. Later, the authors also demonstrated in mouse models of experimental asthma that MSCs activated by AhR efficiently engrafted to injury sites and attenuated allergen-induced lung inflammation (*i.e.*, reduced cell infiltrate and change cytokine profile), mainly *via* TGF- β 1 modulation^[45].

Moreover, it was determined that AhR stimulation in MSCs can also prevent their multipotent differentiation potential. It was shown that treatment with benzo(a)-pyrene (BPs), a specific AhR agonist, markedly inhibited the terminal adipogenic differentiation of MSCs in an AhR-dependent manner, with reduced expression of classical adipogenic markers (FABP4), triglyceride enzymes (G3PDH) and adipogenic transcription factors (PPAR γ and CEBP β)^[31]. Despite the decreased expression of AhR in differentiated MSCs, the expression of its target gene *CYP1B1* remained elevated, indicating that AhR activation was fully functional during adipogenesis. Later, this same study demonstrated that the use of α -NF, an AhR antagonist, abrogated the AhR-mediated inhibition of MSC adipogenesis^[31]. Complementarily, another report demonstrated that BP treatment inhibited adipocyte differentiation *in vitro* by down-regulating the PPAR γ signal and increased the expression of cytochrome P450 (CYP1A1) in canine MSCs^[46]. In addition, it was detected *in vitro* that TCDD-stimulated MSCs suppressed the mRNA levels of osteoblastic markers (*i.e.*, *Runx2*, *Ocn* and *Alp*) in a dose-dependent manner through a process mediated by the inhibition of β -catenin expression. Later, similar observations in MSCs derived from inflamed collagen-induced arthritis mice (a possible environment for AhR activation) showed elevated nuclear expression and translocation

of AhR and, in consequence, inhibition of osteogenesis-associated genes as well as reduced β -catenin expression^[47]. In fact, an additional study verified that AhR activation by BPs inhibited the MSC mesodermal differentiation, and when these activated MSCs were applied in a mouse model of bone fracture, the tibial ossification was affected mainly *via* SMAD-dependent (*e.g.*, TGF- β 1/SMAD4) and SMAD-independent (*e.g.*, TGF- β 1/ERK/AKT) signals^[48]. Therefore, these results illustrate that the adipogenesis and osteogenesis signalling pathways are also potential targets for AhR regulation in MSCs.

Finally, another group found that the activation of MSCs through kynurenine, a natural AhR agonist, can enhance its immunosuppressive response. The authors detected that MSCs stimulated by kynurenine were more effective in suppressing *in vitro* lymphocyte proliferation than MSCs stimulated by IFN- γ and TGF- β separately. Further, the analysis of cytokines in the supernatants of lymphocyte/MSC co-cultures demonstrated that the combination of kynurenine with IFN- γ and TGF- β stimuli significantly reduced IL-6 and IL-17 secretion. In line with these findings, the authors also found that the combination of three effector stimuli (IFN- γ , TGF- β and kynurenine) promoted the overexpression of important immunomodulatory genes in MSCs (*e.g.*, iNOS, IDO, COX2, HO-1, PGE-2, LIF and PD-L1). Later, when these triple-activated MSCs were used in the treatment of an experimental model of GVHD, the stimulated MSCs substantially decreased the inflammation and tissue injury score at a more significant level than normal unstimulated MSCs^[49].

Altogether, these recent studies suggest that AhR activation can substantially modulate the function of MSCs by mechanisms associated with: (1) The induction of the death signal in pro-inflammatory cells, *i.e.*, pre-B cells; (2) the suppression of pro-inflammatory cytokines, *i.e.*, IL-6; (3) the improvement of migration and regenerative potential in acute inflammatory models, *i.e.*, asthma and GVHD; (4) the inhibition of mesodermal differentiation, *i.e.*, adipogenesis and osteogenesis; and (5) the up-regulation of global immunosuppression, *i.e.*, the up-regulation of immunoregulatory genes (Figure 1).

CONCLUSION

The immunosuppressive properties of MSCs are of great interest for cellular therapy; however, randomized double-blind clinical studies have not shown clear benefits to date^[42,50]. This inconclusive large-scale clinical result may be associated with the variety of cytokines/agonists in the distinct environments that MSCs encounter *in vivo*. In this context, the molecular mechanisms involved in the reparative status of MSCs through the activation of sensitive immune-associated receptors are so far unclarified, and, therefore, they are indispensable parameters for investigation. Thus, MSC activation is currently considered a *sine qua non* condition for MSCs and their bioproducts (*i.e.*, trophic factors and

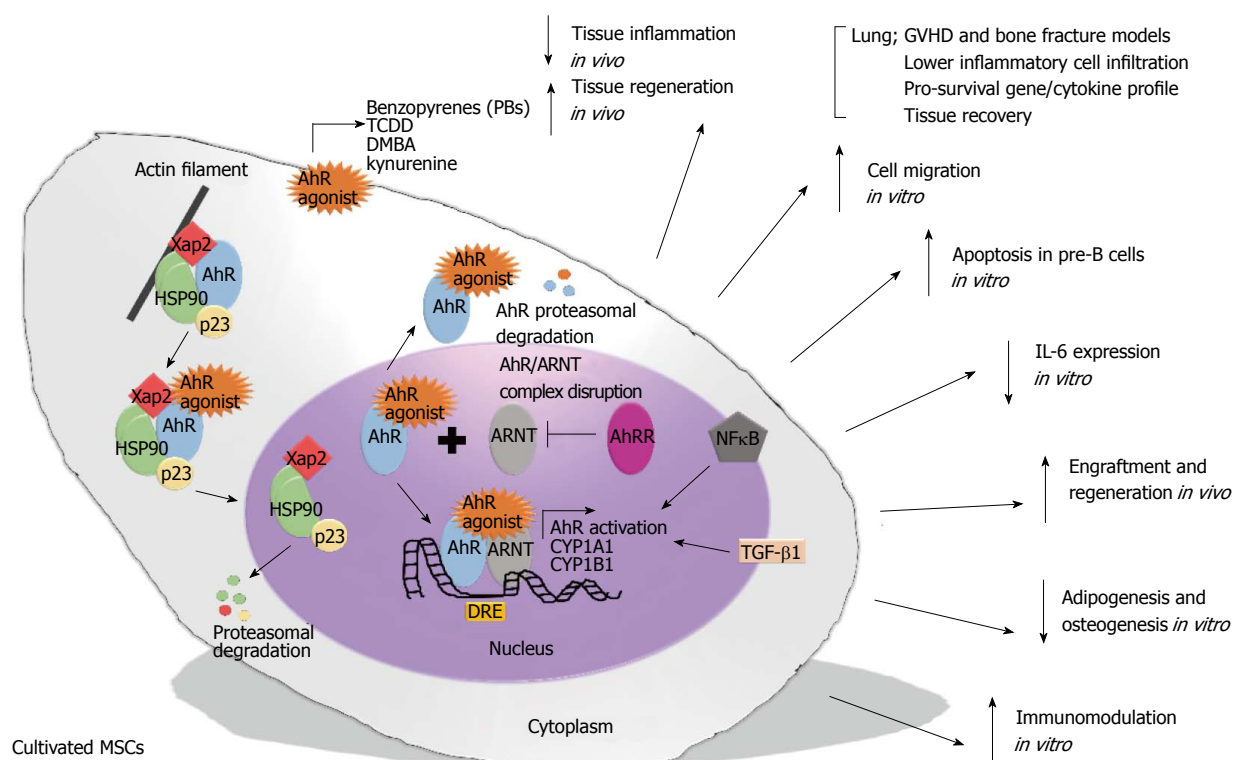


Figure 1 Illustration demonstrating a hypothetical summary of the potential effect of aryl-hydrocarbon receptor activation on multipotent mesenchymal stromal cell function. AhR-mediated MSC activation occurs by a cascade of events that substantially modulate the function of the MSCs by mechanisms associated with: (1) The induction of death signalling in pro-inflammatory cells, *i.e.*, pre-B cells; (2) suppression of pro-inflammatory cytokines, *i.e.*, IL-6; (3) the improvement of migration and regenerative potential in acute inflammatory models, *i.e.*, asthma; (4) the inhibition of mesodermal differentiation, *i.e.*, adipogenesis; and (5) the up-regulation of global immunosuppression, *i.e.*, the up-regulation of immunoregulatory genes. AhR: Aryl-hydrocarbon receptor; MSC: Multipotent mesenchymal stromal cell.

microvesicles) to exert their immunoregulatory response.

Considering this perspective, the quality of the immunoregulatory profile of MSCs can be considerably improved when these cells are exposed to sufficient levels of sensitive ligands (*i.e.*, cytokines/growth factors). On the other hand, MSCs not subjected to pre-stimulation tend to decrease or lose their intrinsic immunosuppressive potential, promoting an undesired inflammatory response^[49]. In this context, we hypothesized that the optimal immunomodulatory potential of MSCs can be obtained by establishing a steady regulatory phenotype in MSCs using precise MSC-responsive ligands as AhR agonists. Thus, the activation of AhR in MSCs should be extensively explored as a mechanism in relevant pre-clinical and experimental studies, in the attempt to improve the applicability of MSCs in a set of degenerative and immunological diseases.

However, questions regarding the mechanisms of the MSC immunoregulatory response remain inconclusive. In this sense, MSC immunoregulation can vary among species, for instance, IDO up-regulation in MSCs is better described in humans, while inducible nitric oxide synthase (iNOS) is a key regulatory enzyme in mouse MSC immunomodulation^[49]. In addition, the elucidation of the cross-talk between AhR agonists and other sensitive molecules (*e.g.*, IFN γ , TGF- β , TNF- α ,

LPS and others) is a detrimental factor in applying the immunosuppressive response of MSCs. Moreover, the influence of MSCs in another set of experimental models is also important to consider. In line with this purpose, Aleman *et al.*^[49] (2015) reported that kynurenine, in combination with other effector stimuli (IFN γ and TGF- β), can induce elevated IDO, COX2, iNOS, and PGE-2 expression in MSCs and, at the same time, reduce the expression of EGFR, MHC II and IL-6. Thus, further investigations should focus on identifying the major components that trigger the activation of the AhR signal and its cross-talk with other signalling pathways, to precisely understand the regulatory mechanism of AhR influence on MSC function. In line with this goal, aspects of this mechanism have begun to be investigated, such as the impact of AhR activation on MSC adipogenesis or osteogenesis; nevertheless, the specific AhR-dependent signalling pathways by which AhR agonists affect MSC-associated mesodermal differentiation also remain to be determined.

In conclusion, we hope that the findings discussed here in this minireview will contribute to better comprehension of the major mechanisms behind MSC immunoregulation and provide a basic background for the development of innovative studies focused on the molecular cascade associated with AhR activation in MSCs. In summary, the study of AhR activation

can promote new insights for the better investigation of molecular signalling pathways associated with the regenerative and immunosuppressive potential of MSCs, and consequently, these studies will support the development of potential MSC-derived therapies for a wide variety of immune-associated disorders.

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Basic Study

Murine hepatocellular carcinoma derived stem cells reveal epithelial-to-mesenchymal plasticity

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Abstract**AIM**

To establish a model to enrich and characterize stem-like cells from murine normal liver and hepatocellular carcinoma (HCC) cell lines and to further investigate stem-like cell association with epithelial-to-mesenchymal transition (EMT).

METHODS

In this study, we utilized a stem cell conditioned serum-free medium to enrich stem-like cells from mouse HCC and normal liver cell lines, Hepa 1-6 and AML12, respectively. We isolated the 3-dimensional spheres and assessed their stemness characteristics by evaluating the

RNA levels of stemness genes and a cell surface stem cell marker by quantitative reverse transcriptase-PCR (qRT-PCR). Next, we examined the relationship between stem cells and EMT using qRT-PCR.

RESULTS

Three-dimensional spheres were enriched by culturing murine HCC and normal hepatocyte cell lines in stem cell conditioned serum-free medium supplemented with epidermal growth factor, basic fibroblast growth factor and heparin sulfate. The 3-dimensional spheres had enhanced stemness markers such as *Klf4* and *Bmi1* and hepatic cancer stem cell (CSC) marker *Cd44* compared to parental cells grown as adherent cultures. We report that epithelial markers *E-cadherin* and *ZO-1* were downregulated, while mesenchymal markers *Vimentin* and *Fibronectin* were upregulated in 3-dimensional spheres. The 3-dimensional spheres also exhibited changes in expression of *Snai1*, *Zeb1* and *Twist* family of EMT transcription factors.

CONCLUSION

Our novel method successfully enriched stem-like cells which possessed an EMT phenotype. The isolation and characterization of murine hepatic CSCs could establish a precise target for the development of more effective therapies for HCC.

Key words: Hepatocellular carcinoma; Hepa 1-6; Cancer stem cells; Cancer initiating cells; Epithelial-to-mesenchymal transition; Cellular plasticity; Epithelial-to-mesenchymal transition transcription factors; AML12

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Core tip: Although existing therapies can initially eliminate the bulk population of a tumor, the stem cell properties of cancer stem cells (CSCs) enable them to survive and repopulate the tumor, resulting in disease relapse. Therefore, elimination of CSCs has the potential to improve patient outcomes and survival. Isolation and characterization of liver CSCs is essential for the selective targeting of this crucial population of cells. We report that the sphere culture method is a more precise and reliable tool for the enrichment of murine stem-like cells which relies on their functional property of anchorage-independent growth.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most

common cancer worldwide affecting one million individuals annually^[1]. HCC is associated with high mortality rates largely due to the development of resistance to chemotherapy or radiotherapy, recurrence after surgery or intra-hepatic metastases^[2]. While treatments such as surgical liver resection and liver transplantation have had a significant impact in early-stage HCC, these treatments have limited efficacy in most patients with advanced stage HCC^[3]. Moreover, Sorafenib, the only available drug for advanced stage HCC has limited efficacy^[4]. A better understanding of the biology of HCC would have a major impact on the management of this disease.

According to the stem cell model of carcinogenesis cancers are initiated and maintained by a rare fraction of cells called cancer stem cells (CSCs) or cancer initiating cells (CICs)^[5,6]. The presence of CSCs with biological properties such as multipotency and self-renewal, similar to those of normal stem cells, was first reported in leukemia and subsequently in diverse malignancies including breast cancer, glioblastoma, prostate cancer, colon cancer and liver carcinoma^[7-13]. CSCs have proven to play a central role in the development, maintenance, metastasis, and recurrence of HCC^[14-16]. Therefore the prospective identification and isolation of CSCs in HCC could generate a better understanding of hepatocarcinogenesis and facilitate the identification of novel druggable targets for development of more efficient therapeutic strategies.

Recent evidence indicates that CSCs may be generated with the reactivation of the developmental epithelial-to-mesenchymal transition (EMT) program, which impacts tumor metastatic potential^[17-19]. EMT describes a reprogramming of epithelial cells that leads to a phenotype switch from an epithelial to a mesenchymal cellular state. This cellular plasticity occurs during normal development as part of processes such as gastrulation and neural crest cell migration. During cancer progression, this phenotype is associated with metastatic dissemination, acquisition of drug resistance and acquisition of CSC state^[20,21]. Whereas the role of EMT in HCC metastasis is well documented, its role in HCC CSC generation is only just emerging^[22].

Although a number of cell surface markers have been identified for the enrichment of HCC derived CSCs, there is no general consensus on the best CSC markers for HCC^[23,24]. We used an alternate method for the enrichment of HCC CSCs based on functional aspect of CSCs. CSCs exhibit anchorage-independent growth and form spheres that possess the capacity for self-renewal and tumorigenicity, when grown in a stem cell conditioned serum-free medium^[25]. Sphere formation assay thus represent a more precise tool for the enrichment of CSCs. This study therefore aimed to enrich stem-like cells from mouse HCC and normal liver cell lines with the goal to better characterize the 3-dimensional spheres. We also sought to examine the relationship between CSCs and EMT.

MATERIALS AND METHODS

Cell lines and cell culture

Murine HCC cell line Hepa 1-6 and normal liver cell line AML12 were procured from American Type Culture Collection (ATCC) and maintained as per ATCC protocols. The cell lines Hepa 1-6 and AML12 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Australia) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Assay Matrix, Australia) and 1% penicillin/streptomycin (P/S) (ThermoFischer Scientific, Australia) and incubated at 37 °C under a humidified atmosphere with 5% CO₂ in air.

3-dimensional sphere enrichment assay

Cells were cultured as 3-dimensional spheres using a stem cell conditioned serum-free medium which is based on a neural stem cell medium^[25]. Stem cell conditioned serum-free medium was prepared by adding 1:1 mixture of DMEM and HAM's F12 medium (Lonza, Australia) supplemented with 4 µg/mL heparin sulfate (Sigma-Aldrich, United States), 1% penicillin/streptomycin (P/S) (ThermoFischer Scientific, Australia), 2% bovine serum albumin (BSA) (Sigma-Aldrich, United States), 20 ng/mL recombinant human epidermal growth factor (rhEGF) (Lonza, Australia) and 10 ng/mL recombinant human basic fibroblast growth factor (rhbFGF) (Lonza, Australia). Briefly, adherent cells were detached and collected following Trypsin-EDTA (ThermoFisher Scientific, Australia) treatment. Cells were washed three times with 50 mL 1 × PBS to remove serum. Cells were counted and seeded at 5000 cells/ml in a T-25 ultra-low-attachment flask (Corning Incorporated, United States) and cultured with stem cell medium at 37 °C in a humidified atmosphere of 5% CO₂ in air.

Enumeration of spheres

Cells were seeded at 2000 cells/well in a 6-well ultra-low-attachment plates (Corning Incorporated, United States) and cultured with stem cell medium. Diameter of 3-dimensional spheroids and number of spheres per culture well were counted on day 5 using an inverted microscope equipped with a digital camera (Olympus DP21, Japan).

RNA extraction and cDNA synthesis

The parental cells were plated at the same density as the sphere cells and on day 5 total cellular RNA was extracted using the Isolate II Bioline RNA synthesis kit (Bioline, Australia) as per the manufacturer's protocol. We performed on column DNAase digestion using RNase-Free DNase at room temperature (20 °C–30 °C) for 15 min in accordance to Bioline RNA synthesis kit instructions. Spectrophotometric quantification using the Nanodrop 2000 c (ThermoFisher, United States) confirmed purity of RNA and absence of DNA in our samples. One micrograms of the extracted RNA was reverse transcribed using the Bioline SensiFAST cDNA

Table 1 List of primers for quantitative reverse transcriptase-PCR

Primer	Sequence (5'-3')
<i>ActB</i> forward	ATGGAGGGGAATACAGCCC
<i>ActB</i> reverse	TTCTTGCAGCTCCTTCGTT
<i>Klf4</i> forward	CAGTGGTAAGGTTTCTCGCC
<i>Klf4</i> reverse	GCCACCCACACTTGACTA
<i>Bmi1</i> forward	TGGTTGTTTCGATGCATTCT
<i>Bmi1</i> reverse	CTTCATTGCTTTTCCGCC
<i>Cd44</i> forward	AGCGGCAGGTTACATTCAAA
<i>Cd44</i> reverse	CAAGTTTGGTGGCACACAG
<i>E-Cadherin</i> forward	AAAAGAAGGCTGCTTGGC
<i>E-Cadherin</i> reverse	GAGGTCTACACCTTCCCGGT
<i>ZO-1</i> forward	CCTGTGAAGCGTCACTGTGT
<i>ZO-1</i> reverse	CGCGGAGAGAGACAAGATGT
<i>Vimentin</i> forward	AGAGAGAGGAAGCCGAAAGC
<i>Vimentin</i> reverse	TCCACTTCCGTTCAAGGTC
<i>Fibronectin</i> forward	ACTGGATGGGGTGGGAAT
<i>Fibronectin</i> reverse	GGAGTGGCACTGTCAACCTC
<i>Snai1</i> forward	AGTGGGAGCAGGAGAATGG
<i>Snai1</i> reverse	CTGTGTCTGCACGACCTGT
<i>Snai2</i> forward	GATGTGCCCTCAGGTTTGAT
<i>Snai2</i> reverse	GGCTGCTTCAAGGACACATT
<i>Zeb1</i> forward	TCATCGGAATCTGAATTTGC
<i>Zeb1</i> reverse	CCAGGTGTAAGCGCAGAAAG
<i>Zeb2</i> forward	TGCGTCCACTACGTGTGCAT
<i>Zeb2</i> reverse	TCTTATCAATGAAGCAGCCG
<i>Twist1</i> forward	CATGTCGCGTCCCACATA
<i>Twist1</i> reverse	TCCATTTTCTCTTCTCTGGA
<i>Twist2</i> forward	GCCTGAGATGTGCAGGTG
<i>Twist2</i> reverse	GTCTCAGCTACGCTTCTCC

synthesis kit (Bioline, Australia).

Quantitative reverse transcriptase-PCR

Following reverse transcription, quantitative reverse transcriptase-PCR (qRT-PCR) was performed using Lo-ROX SYBR Green (Bioline, Australia). Reactions were run in 384-well plates on a ViiA7 Applied Biosystems Real-Time PCR system. Amplification was performed according to a three-step cycle procedure consisting of 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 10 s and extension at 75 °C for 15 s. E-cadherin expression was evaluated using QuantiFast SYBR Green PCR Kit (Qiagen, United States) following the manufacturer's instructions. Amplification was performed according to a two-step cycling procedure consisting of 40 cycles of denaturation at 95 °C for 10 s and combined annealing/extension at 60 °C for 30 s. *Beta-Actin* (*ActB*) was used as an internal control. The primers used are listed in Table 1. Expression levels were normalized to *ActB* and are presented as copies of target gene per 10000 copies of *ActB*, calculated using the formula: $2^{-(CT_{ActB} - CT_{target})} \times 10000$. The copy number values were calculated from a minimum of three independent biological replicates.

Statistical analysis

All experiments were repeated at least three times and representative results are presented. All statistical comparisons of data sets were performed using Student's

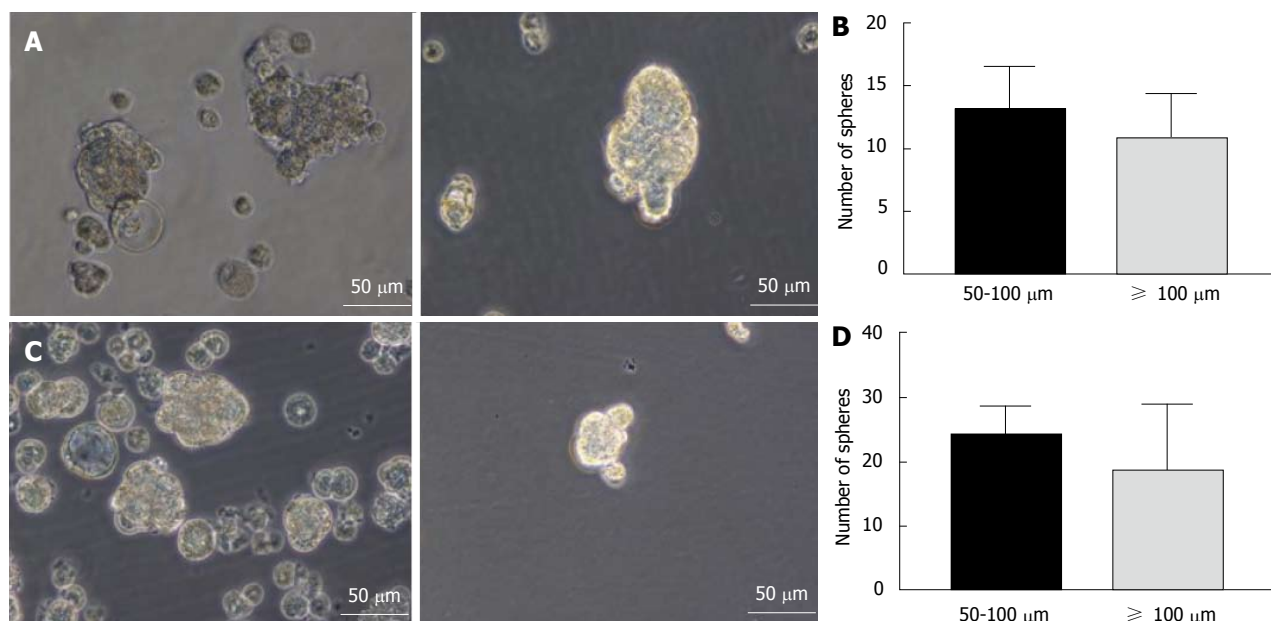


Figure 1 Enrichment of cancer stem cells using the sphere culture method. A: Photomicrographs of Hepa 1-6 3-dimensional spheres on day 5 cultured in stem cell conditioned serum-free medium; B: Hepa 1-6 sphere sizes and numbers were enumerated; C: Photomicrographs of AML12 3-dimensional spheres on day 5; D: AML12 spheres sizes and numbers were enumerated.

s two-tailed *t*-test in GraphPad Prism software version 7.00 (GraphPad Software Inc). Statistical significance was set at ^a*P* < 0.05, ^b*P* < 0.01 and ^c*P* < 0.001.

RESULTS

Mouse HCC and normal liver cells formed anchorage-independent 3-dimensional spheres

Mouse HCC cell line Hepa 1-6 and normal mouse liver cell line AML12 were used for induction of spheres. Both cell lines could form anchorage-independent, non-adherent 3-dimensional spheres when grown in conditioned serum-free culture medium supplemented with rhEGF, rhbFGF and heparin sulfate (Figure 1A and C). Both cell lines formed floating small spheres which eventually form 3-dimensional structures by day 5. No adherent cells were detected. The number of spheres were counted and appeared to be similar in both the cell types (Figure 1B and D).

Embryonic stemness and CSC marker expressions are enhanced in 3-dimensional spheres

With the goal of better characterizing the cells enriched by sphere culture, we examined the expression levels of some stem cell-associated genes important for the proliferation, self-renewal and differentiation of stem cells. As controls, the parental cells were plated as adherent cultures at the same density as the spheres. On day 5 RNA was extracted from 3-dimensional sphere cultures and adherent cultures. qRT-PCR analysis revealed markedly elevated expression of embryonic stem cell-associated genes Kruppel like factor 4 (*Klf4*) and Bmi1 polycomb ring finger oncogene (*Bmi1*) in Hepa 1-6 spheres compared with parental cells (Figure 2A and

B). *Cd44*, a cell surface adhesion molecule which has been used as a CSC marker in HCC showed significantly increased expression in Hepa 1-6 spheres compared with adherent parental cells (Figure 2C). Similarly, AML12 derived 3-dimensional spheres also expressed significantly higher mRNA levels of *Klf4* and *Bmi1* compared with the adherent AML12 population (Figure 2D and E). Higher expression of *Cd44* was detected in spheres from AML12 compared with the parental cells (Figure 2F). These results indicate that the conditioned stem cell serum-free medium is a precise tool for the selective enrichment of hepatic mouse stem-like cells.

CSC and EMT phenotypes are linked in 3-dimensional spheres

To elucidate whether there were connections between the spheres and EMT phenotype, we assessed the EMT characteristics of the 3-dimensional spheres from Hepa 1-6 and AML12. At the molecular level, EMT is characterized by a series of coordinated changes including down-regulation of the adherens junction molecule *E-cadherin* and tight junction molecule Zonula occludens-1 (*ZO-1*) and upregulation of *Vimentin*, an intermediate filament and *Fibronectin*, a key molecule of extracellular matrix. We observed that the expression of classical epithelial marker genes, *E-cadherin* and *ZO-1* were significantly downregulated in 3-dimensional spheres from both Hepa 1-6 and AML12 compared with parental cells (Figure 3). These 3-dimensional spheres also exhibited the characteristic features of a mesenchymal phenotype with high expression of *Vimentin* and *Fibronectin* (Figure 4). These findings suggest that the stem cell phenotype is closely linked with an EMT phenotype.

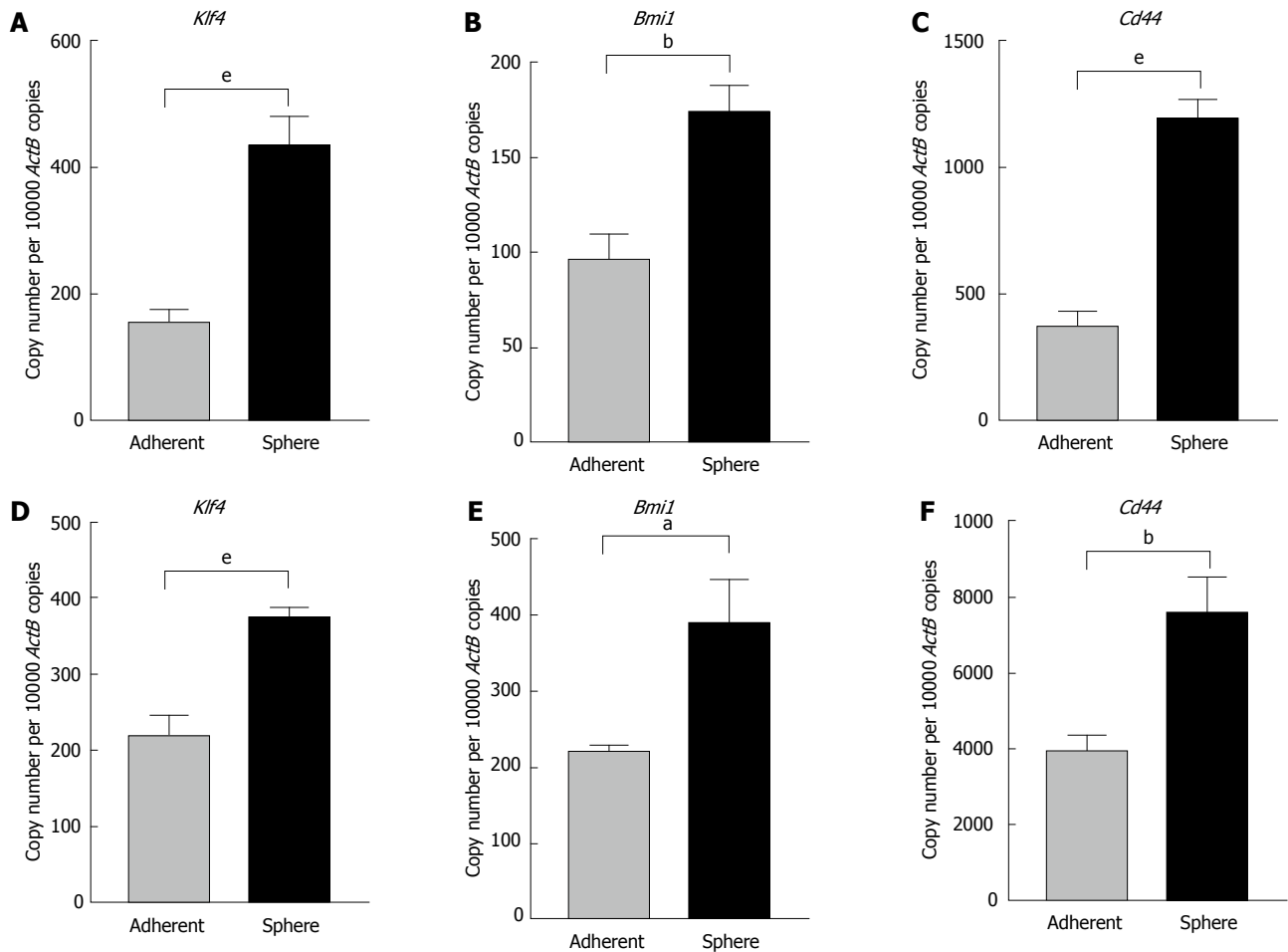


Figure 2 Three-dimensional spheres expressed high stemness and cell surface cancer stem cell markers. qRT-PCR analysis revealed higher expression of A: *Klf4*, B: *Bmi1* and C: *Cd44* in Hepa 1-6 3-dimensional spheres compared with Hepa 1-6 grown as adherent cells. qRT-PCR analysis revealed higher expression of D: *Klf4*, E: *Bmi1* and F: *Cd44* in AML12 3-dimensional spheres compared with AML12 grown as adherent cells. Values are mean \pm SEM of three experiments in triplicate ($^aP < 0.05$, $^bP < 0.01$, $^eP < 0.001$). qRT-PCR: Quantitative reverse transcriptase-PCR; ActB: Beta-Actin.

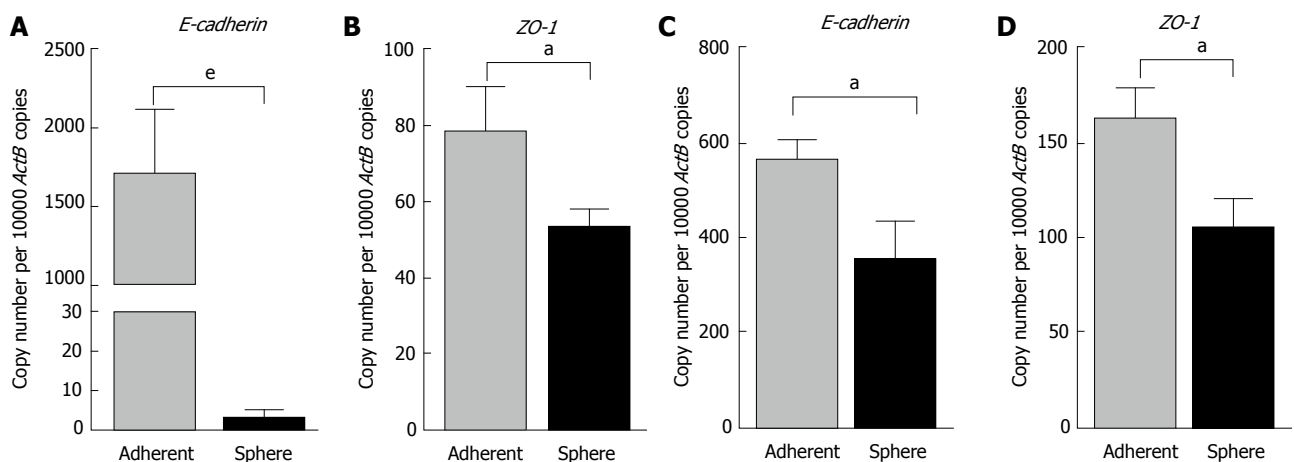


Figure 3 3-dimensional spheres decreased expression of epithelial markers. qRT-PCR analysis revealed downregulation of A: *E-cadherin* and B: *ZO-1* in Hepa 1-6 CSCs compared with Hepa 1-6 grown as adherent cells. qRT-PCR analysis revealed lower expression of C: *E-cadherin* and D: *ZO-1* in AML12 CSCs compared with AML12 grown as adherent cells. Values are mean \pm SEM of three experiments in triplicate ($^aP < 0.05$). qRT-PCR: Quantitative reverse transcriptase-PCR; ActB: Beta-Actin.

Core EMT transcription factors are enhanced in 3-dimensional spheres

To further confirm the occurrence of EMT process in

CSCs we examined the expression levels of core EMT transcription factors that govern cellular plasticity. In Hepa 1-6 spheres we observed significant upregulation

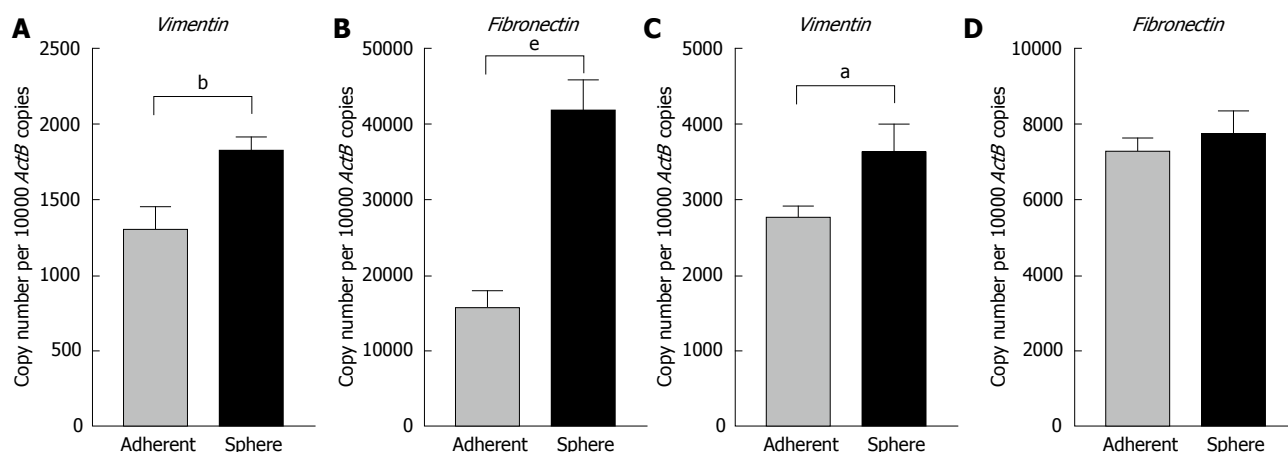


Figure 4 Three-dimensional spheres have higher expression of mesenchymal markers. qRT-PCR analysis revealed upregulation of A: *Vimentin* and B: *Fibronectin* in Hepa 1-6 3-dimensional spheres compared with Hepa 1-6 grown as adherent cells. qRT-PCR analysis revealed higher expression of C: *Vimentin* and D: *Fibronectin* in AML12 3-dimensional spheres compared with AML12 grown as adherent cells. Values are mean \pm SEM of three experiments in triplicate ($^aP < 0.05$, $^bP < 0.01$, $^eP < 0.001$). qRT-PCR: Quantitative reverse transcriptase-PCR; ActB: Beta-Actin.

of *Snai* family of transcription factors (*Snai1* and 2), Zinc-finger E-box-binding homeobox family of transcription factors (*Zeb1* and 2) and helix-loop-helix *Twist* family of transcription factors (*Twist1* and 2) compared with adherent parental cells (Figure 5). We observed a significant increase in the *Zeb* family of transcription factors in AML12 3-dimensional spheres compared with parental cells. AML12 3-dimensional spheres showed downregulation of *Snai2*, *Twist1* and *Twist2* RNA levels (Figure 6). This raises the possibility that distinct family of transcription factor may enable maintenance of CSC cellular plasticity in different cell types. Together, these features of EMT strongly suggest a possible relationship of EMT with the hepatic stem-like cell phenotype.

DISCUSSION

Worldwide, HCC, a primary liver cancer is one of the most common malignancies with a poor outcome^[2]. Non-resectable advanced stage HCC remains an incurable disease for which novel therapies are urgently needed. Accumulating evidence suggests that CSCs play an important role in HCC tumorigenicity and the reactivation of EMT process has been implicated in the generation of CSCs^[22]. The CSC field has experienced rapid advances in the past decade and a number of strategies have been applied to identify and harvest them^[12-14,26-28]. Several markers have been proposed for the identification of CSCs in HCC, but not all are uniformly expressed in all CSC populations and single markers have been deemed insufficient to represent the real CSC phenotype^[24]. Alternately, the sphere culture method, which is not dependent on markers, has been increasingly utilized in various tumors, including HCC for isolating, enriching, maintaining or expanding the potential CSC subpopulations^[25,29-32]. To our knowledge, this is the first time that murine HCC and normal hepatocyte cell lines have been examined for sphere

forming capacity, enrichment of stem-like cells and occurrence of epithelial-mesenchymal plasticity.

Enrichment and characterization of murine derived CSCs provides a better understanding of how these CSCs interact with the CSC niche environment and host immune system in order to form a tumor and are indispensable for the development of new therapies for the elimination of CSCs. In HCC, the majority of studies of CSCs have utilized patient-derived material or established human tumor cell lines inoculated into immunocompromised mice^[29,31,32]. The immunocompromised mouse microenvironments do not recapitulate the microenvironment in a human patient with naturally occurring cancer and have limited value in assessing therapies targeting CSCs. Moreover, the ability of cells to grow in immunocompromised mice does not distinguish CSCs from non-CSCs, as it demonstrates selection for cells that can best adapt to growth in murine tissue, and therefore might not represent a true approximation of CSCs^[25]. We have previously demonstrated that immunocompetent syngeneic models allow for interactions of the recipient mouse host immune system with CSCs, a situation that more closely models cancer in humans^[25]. Future studies are needed to address whether mouse HCC derived CSCs are able to initiate tumors in syngeneic immunocompetent mice compared with the parental counterparts.

Our stem cell enrichment medium comprised of serum free media supplemented with rhEGF, rhbFGF and heparin sulfate, while others have previously used media supplements such as B27, leukemia inhibitory factor, N-acetyl-L-cysteine and neural survival factor for enriching human HCC CSCs^[29,30]. Our finding that murine 3-dimensional spheres had enhanced expression of stem cell markers namely, *Klf4*, *Bmi1* and *Cd44* lends credence to the use of the sphere culture model for CSC enrichment. Positive expression of *KLF4* was correlated with tumor relapse and a poor prognosis in patients with HCC^[33]. *CD44* expression was highly correlated with

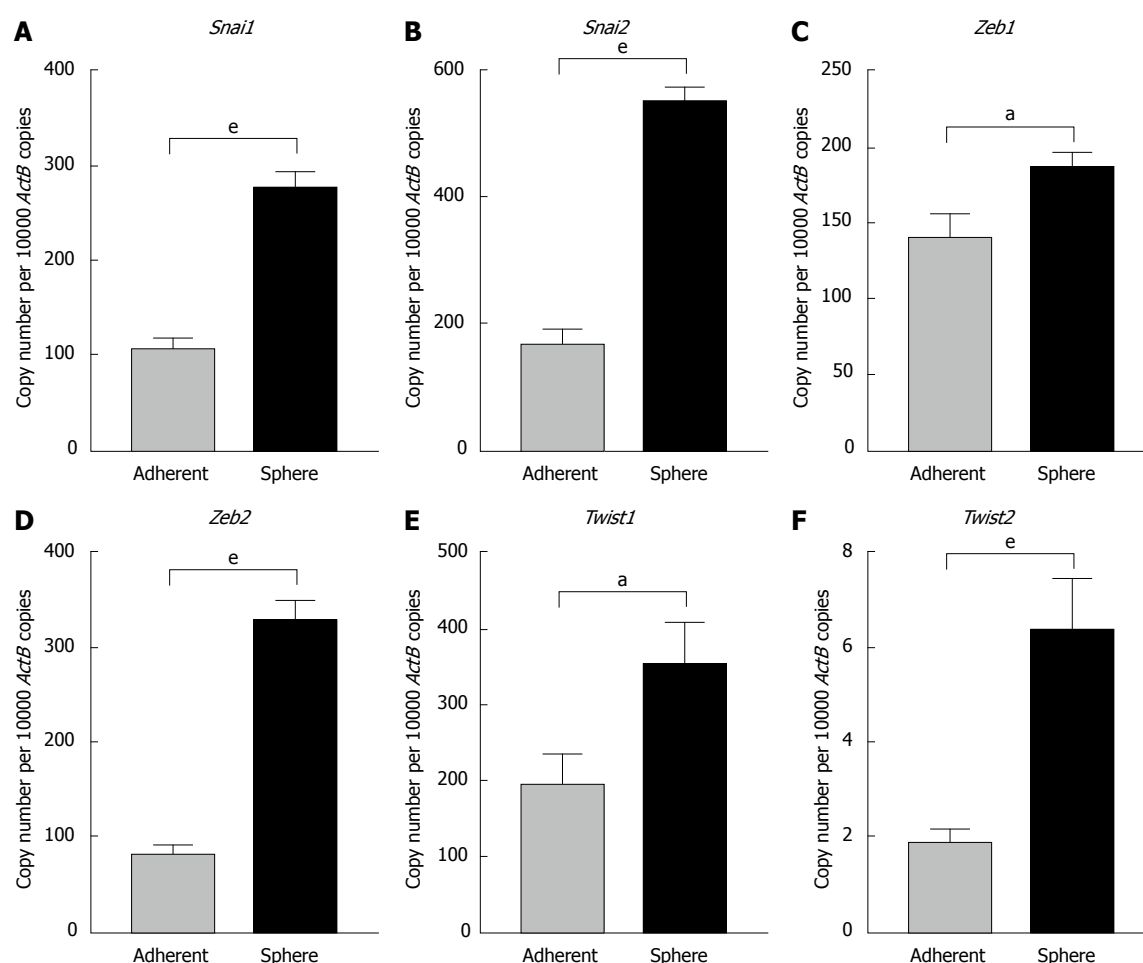


Figure 5 Expression of putative epithelial-to-mesenchymal transition transcription factors in Hepa 1-6 3-dimensional spheres. qRT-PCR analysis revealed upregulation of A: *Snai1*, B: *Snai2*, C: *Zeb1*, D: *Zeb2*, E: *Twist1*, and F: *Twist2* in Hepa 1-6 3-dimensional spheres compared with Hepa 1-6 grown as adherent cells. Values are mean \pm SEM of three experiments in triplicate ($^{\circ}P < 0.05$, $^{\circ}P < 0.001$). qRT-PCR: Quantitative reverse transcriptase-PCR; ActB: Beta-Actin.

decreased overall survival in HCC patients^[34] while high *BMI1* expression was associated with a poor prognosis in HCC patients^[35].

Finally we demonstrate a striking association between the expression of CSC and EMT markers. The biologic link between EMT phenotypes and CSCs has recently been evidenced in many types of cancer, including HCC^[3,22]. *E-cadherin* functions as a key gatekeeper of the epithelial state. Loss or downregulation of *E-cadherin* has been considered to be a hallmark of EMT^[20,21]. In our study, 3-dimensional spheres demonstrated downregulation of *E-cadherin* and *ZO-1*. We also found that the 3-dimensional spheres exhibited high *Vimentin* and *Fibronectin*, the phenotypes of mesenchymal cells that have more aggressive biological behaviour. Most notably, we found elevation of core EMT transcription factors in 3-dimensional spheres. Downregulation of *E-cadherin* is often mediated by core EMT-controlling transcription factors of *Snai*, *Zeb* and *Twist* families which have recently been molecularly linked to self-renewal programs^[36]. AML12 have yielded mixed results for EMT transcription factors in spheres with downregulation of *Twists* and *Snai2*. This indicates apparent cell type-

specific differences and the cause for this variance in transcription factor expression remains elusive and warrants further investigation. Taken together, our findings indicate that EMT transcription factors such as *Snai1*, *Zeb1* and 2 may provide opportunities for therapeutic targeting of CSC *via* blocking EMT. An in-depth investigation of crosstalk of stemness with EMT is essential for a better understanding of tumor progression in HCC. It is clear that further studies of CSC characterization will be critical to better understand plasticity and the mediators of phenotype switching as contributors to HCC initiation, progression, treatment failure and disease relapse. As a central player in these processes, EMT transcription factors may well serve as druggable targets in strategies to better treat HCC.

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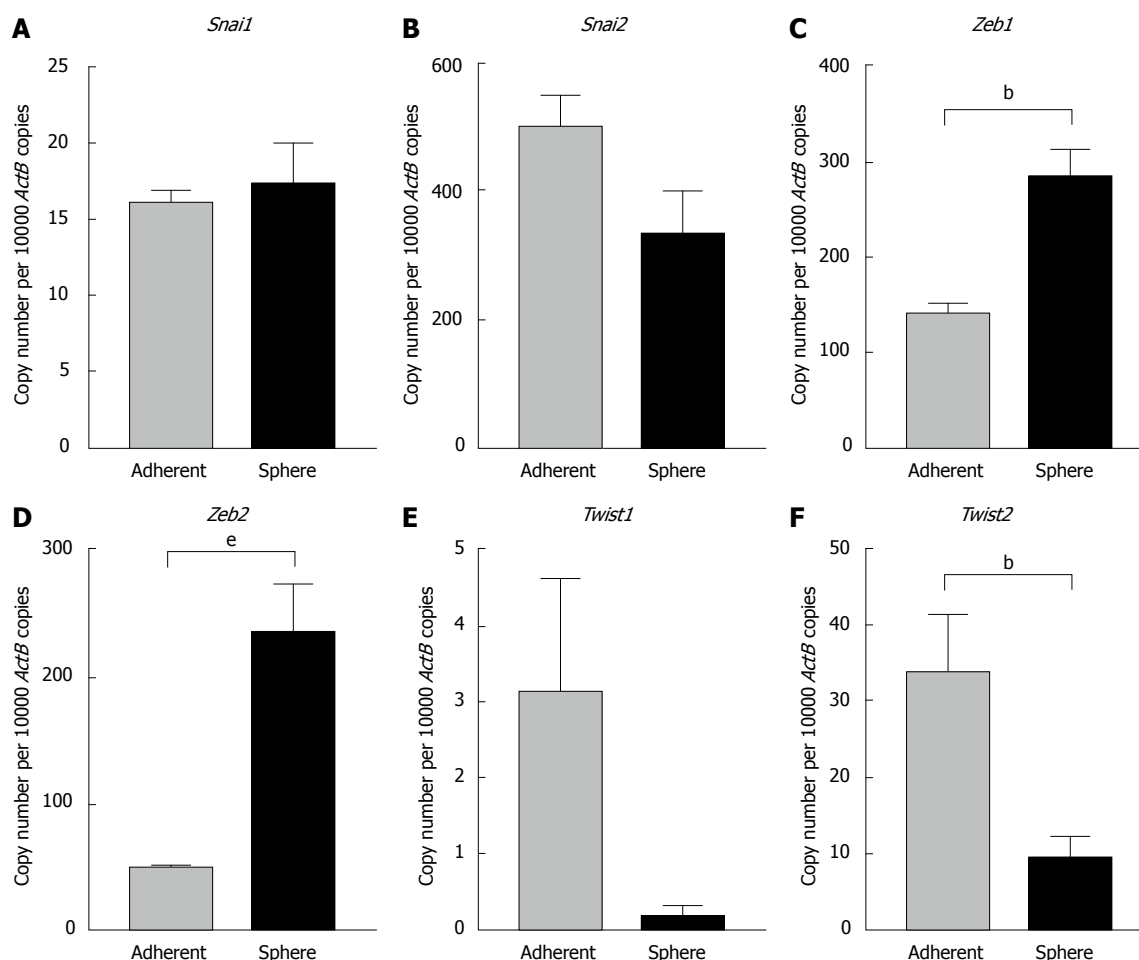


Figure 6 Expression of putative epithelial-to-mesenchymal transition transcription factors in AML12 3-dimensional spheres. qRT-PCR analysis revealed upregulation of A: *Snai1*, C: *Zeb1*, D: *Zeb2* and downregulation of B: *Snai2*, E: *Twist1*, and F: *Twist2* in AML12 3-dimensional spheres compared with AML12 grown as adherent cells. Values are mean \pm SEM of three experiments in triplicate ($^bP < 0.01$). qRT-PCR: Quantitative reverse transcriptase-PCR; ActB: Beta-Actin.

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COMMENTS

Background

Cancer stem cells (CSCs) have proven to play a central role in the development, maintenance, metastasis, and recurrence of hepatocellular carcinoma (HCC). Therefore the prospective identification and isolation of CSCs in HCC could generate a better understanding of hepatocarcinogenesis and facilitate the identification of novel druggable targets for development of more efficient therapeutic strategies.

Research frontiers

Although a number of cell surface markers have been identified for the enrichment of HCC derived CSCs, there is no general consensus on the best CSC markers for HCC. The authors used an alternate method for the enrichment of HCC CSCs based on functional aspect of CSCs.

Innovations and breakthroughs

To the knowledge, this is the first time that murine HCC and normal hepatocyte cell lines have been examined for sphere forming capacity, enrichment of stem-like cells and occurrence of epithelial-mesenchymal plasticity.

Applications

The authors' findings indicate that EMT transcription factors such as *Snai1*, *Zeb1* and 2 may provide opportunities for therapeutic targeting of CSC via

blocking EMT. An in-depth investigation of crosstalk of stemness with EMT is essential for a better understanding of tumor progression in HCC. It is clear that further studies of CSC characterization will be critical to better understand plasticity and the mediators of phenotype switching as contributors to HCC initiation, progression, treatment failure and disease relapse.

Terminology

CSC is cancer stem cells which have biological properties such as multipotency and self-renewal, similar to those of normal stem cells. EMT describes epithelial-to-mesenchymal transition, a reprogramming of epithelial cells that leads to a phenotype switch from an epithelial to a mesenchymal cellular state.

Peer-review

This manuscript is interesting, presenting a feasible method for concentrating a stem-like population from hepatic cancer cells by extending their previously reported technique for enriching a cancer-initiating population from lung cancer cell lines.

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Tracing and targeting cancer stem cells: New venture for personalized molecular cancer therapy

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Abstract

Tumors consist of a mixture of heterogeneous cell types. Cancer stem cells (CSCs) are a minor sub-population within the bulk cancer fraction which has been found

to reconstitute and propagate the disease and to be frequently resistant to chemotherapy, irradiation, cytotoxic drugs and probably also against immune attack. CSCs are considered as the seeds of tumor recurrence, driving force of tumorigenesis and metastases. This underlines the urgent need for innovative methods to identify and target CSCs. However, the role and existence of CSCs in therapy resistance and cancer recurrence remains a topic of intense debate. The underlying biological properties of the tumor stem cells are extremely dependent on numerous signals, and the targeted inhibition of these stem cell signaling pathways is one of the promising approaches of the new antitumor therapy approaches. This perspective review article summarizes the novel methods of tracing CSCs and discusses the hallmarks of CSC identification influenced by the microenvironment or by having imperfect detection markers. In addition, explains the known molecular mechanisms of therapy resistance in CSCs as reliable and clinically predictive markers that could enable the use of new targeted antitumor therapy in the sense of personalized medicine.

Key words: Cancer stem cells; Cancer recurrence; Cancer therapy; Combination therapy; Chemotherapy; Radiation therapy; Immunotherapy

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Core tip: Cancer stem cells (CSCs) are small subpopulation of the tumor that can survive from conventional treatment, escape from the immune system and can cause recurrence of cancer disease. Therefore, any attempt in detection and selective therapeutic targeting of CSCs will ultimately lead to better cancer treatments and can play an important role in reducing the cancer related mortalities. This review highlights the trends and approaches in CSC tracing, isolating, characterizing and targeting, which are key strategies for a novel personalized molecular cancer therapy.

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INTRODUCTION

Cancer originates from deregulation of growth and resistance to apoptosis of transformed cells that acquire proliferative and metastatic capacity. While only a few genetic and epigenetic alterations can initiate the malignant transformation of healthy cells, clinically visible tumors are extraordinarily complex structures with cancer cells displaying a large number of mutations and altered gene expression^[1]. The hierarchical model of tumor organization represents a similar, albeit distorted, arrangement of the tumor cells, as are their tissues of origin. The stem cell population is positioned at the top of the cell hierarchy and has the ability to self-renew and multilineage differentiate to progenitors or differentiated cell types whose proliferation capacity is restricted^[2,3].

The theory of the cancer stem cell (CSC) was postulated in the 1970s and was confirmed experimentally by the isolation of tumor-initiating cells using cellular/molecular biomarkers that allowed the isolation of CSCs in acute myeloid leukemia^[4]. Further, CSC has been demonstrated in a variety of solid tumors such as tumors in brain, colorectal, head and neck, liver, lung, mammary glands, pancreatic prostate carcinomas, melanoma and hematopoietic malignancies (e.g., myeloid or lymphoid leukemia)^[5-7]. Cell lines derived from these tumors also contain CSCs and tumor precursor cells, which represent a promising model for cancer stem cell research^[1]. The functional characterization of CSCs revealed that these cells represent a small subpopulation of the tumor that can survive from conventional treatment, escape from the immune system and therefore can cause recurrence of cancer disease. Therefore, CSCs are driving force of tumorigenesis and metastases (Figure 1). According to the concept of a stem cell, it is assumed that even a few surviving CSCs after tumor therapy, is sufficient to form a new tumor^[8].

In each cancer cell clone, which is characterized by harboring different combinations of mutations or genetic alterations, the processes of self-renewal, and differentiation occur differently based on the type of genetic lesions^[9]. Nevertheless, significant similarities between normal and tumorigenic, experimentally identified stem cells could be expected. Both stem cell types (normal or cancerous) are rarely active, dependent on a specific microenvironment (so-called "stem cell-niche") and have a number of self-protection mechanisms^[2]. This niche enables a dynamic interaction between stem cells and surrounding cells including immune cells ("immune-niche"), cytokines and chemokines that regulates maintenance, quiescence, self-renewal and differentiation of stem cells to provide an

optimal stem cell-supporting setting. What contributes to formation of the niche for tumor stem cells is the subject of intensive research^[10]. Normal stem cells are more microenvironment dependent in order to get dynamic input to balance between activation and differentiation or self-renewal and quiescence "extrinsic factors"^[11,12]. Although CSCs can represent more autonomous regulatory characterization "intrinsic factors", similar concept of stem cell niche support could also hold for them^[13]. The majority of studies using the isolated CSCs, shows the dominant effect of intrinsic factors on CSC regulation. While, other studies propose a role for the CSC niche^[12]. This model suggests that less malignant tumors may have more demand on the stem cell-niche but upon cancer progress this dynamic interplay might be weakened or even diminished^[14].

An inducer of the stem cell phenotype is hypoxia^[15-17]. The self-protection mechanisms are due to the expression of numerous proteins, which reduce the effects of genotoxic xenobiotics. These include the members of efflux pumps, such as ABCB1-MDR1, ABCC1-MRP1 and ABCG2-BCRP, other specific detoxification systems, such as aldehyde dehydrogenase and increased DNA repair capacity. The symmetric cell division and asymmetric distribution of the DNA can also be regarded as part of stem cell self-protection mechanisms^[9]. For the tumor stem cells, the existence of the same mechanisms is a crucial cause of their therapeutic resistance.

In addition to hypoxia as a triggering factor, growth factors play an important role, leading to epithelial-mesenchymal transition (EMT) in cells. It is shown a high-level regulation of stem cell markers after the induction of EMT in normal epithelial cells of the breast gland tissue and in mammary carcinoma cells^[18]. One of the EMT effects can be the induction of the stem cell phenotype^[18].

Numerous findings could show that routine tumor therapy approaches (classical chemotherapy or radiation therapy) and even the majority of currently used targeted antitumor drugs, so-called biological therapy, have little effect on the tumor stem cells even in chemo- or radio-sensitive tumors^[19]. While, the stationary tumor stem cells largely retain their epithelial character and are therefore responsible for the primary tumor growth or recurrence, the migrating tumor stem cells exhibit ability for invasion and distant metastasis. This highlights the above-mentioned plasticity of the tumor stem cells (Figure 1).

THERAPY RESISTANCE IN CSCs

A small number of immortal cells within the bulk tumor with a character of CSC causes the chemo/radiotherapy resistance. Such cells with stem cell characteristics, seem to grow aggressively and metastasize easily. It is not yet clear how CSCs are formed, whether they develop from tissue stem cells or are formed from differentiated cells by recovering embryonic properties. Chemotherapeutic agents and radiotherapy mainly destroy dividing cells^[20].

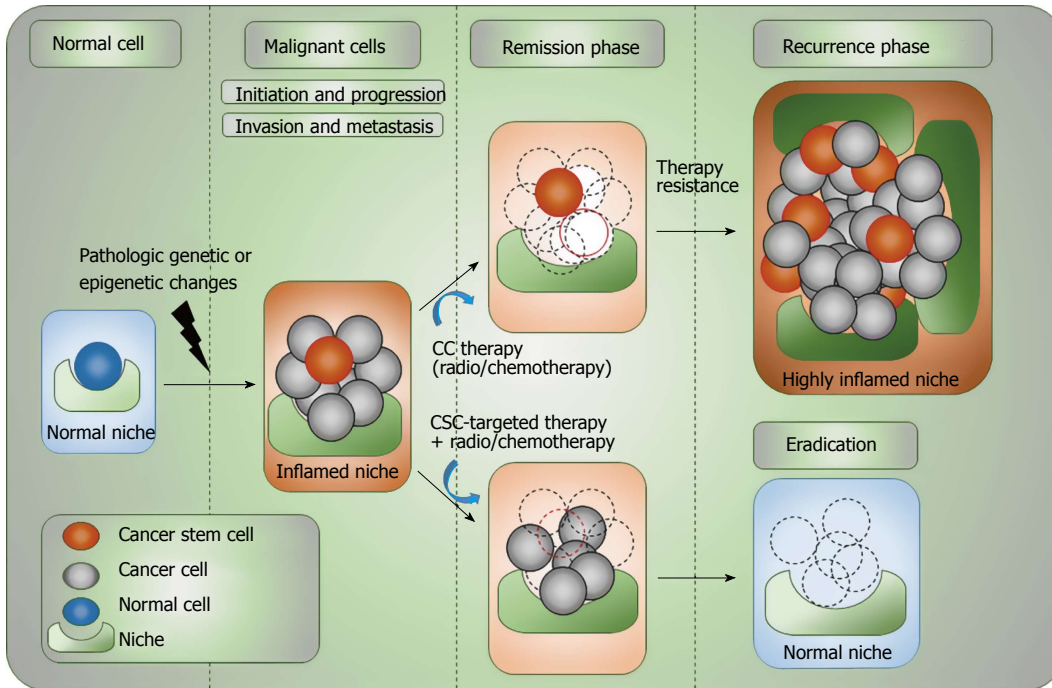


Figure 1 Complex organization of cancer initiation, progress, remission and relapse. CSCs are capable of undergoing extensive cell proliferation after acquiring different pathologic genetic/epigenetic changes while retaining their stemness and giving rise to differentiated progenies. Acquiring further genetic/epigenetic changes during different stages of tumor progression will evolve CSCs, but this may also be advanced through having dynamic interplay with the stem cell-niche. Both CSCs and non-CSCs can be found at the invasive front of primary tumors, which is linked to the process of EMT. However, only CSCs are capable of surviving from immune-surveillance or conventional tumor therapies and are able to give rise to distant metastasis or cause cancer recurrence. The potential eradication of tumor cells and CSCs can be resulted only upon combination targeted therapeutic approaches. Tumor stem cell-targeting drugs should be able to prolong the efficacy of cytotoxic tumor therapy and reduce the recurrence risk. CSC: Cancer stem cell; CC: Cancer cell; NC: Normal cell; EMT: Epithelial-mesenchymal transition.

Since CSCs are particularly dormant, in one hand they are not detected by the routine screening measures, and in the other hand, they are positively selected upon the routine therapy approaches.

MOLECULAR MECHANISMS OF THE THERAPY RESISTANCE OF CSCs

Central regulators of the cellular response to DNA damage are checkpoint kinases 1 and 2 (Chk1/2), which are activated after genotoxic stress and stop cell proliferation to allow DNA repair. Activation of Chk1 as a response to DNA damage by ionizing radiation or chemotherapy agents can be detected preferentially in CD133⁺ glioblastoma precursor cells^[21]. By pharmacological inhibition of Chk1, it was possible to increase the sensitivity of CD133⁺ glioblastoma precursor cells against therapy^[21].

An efficient inactivation of reactive oxygen species (ROS) is another feature of CSCs. The excessive production of ROS under chemo/radiotherapy leads to a cell damage because of its interaction with DNA and proteins and triggering the cell death. In some tumors, including mammary carcinoma and gastrointestinal carcinoma, fewer amounts of ROS were detected in CSCs with a simultaneously increased amount of free-radical scavenger compared to the cell populations without CSC phenotype^[22]. In addition, the expression of stem cell marker CD44 in tumor cells was associated with an

increased expression of the glutathione as a free-radical scavenger^[23,24]. Pharmacologically induced reduction in the concentration of free-radical scavenger in tumor cells can significantly increase their sensitivity to the chemo/radiotherapy^[25]. It remains unclear whether the increased CD44 expression as a biomarker is suitable for the detection of ROS-resistant CSCs and thus can identify patients who can benefit from therapy with inhibitors of free-radical scavengers in combination with the chemo/radiotherapy.

Another factor contributing to the chemo/radiotherapy resistance of CSCs is hypoxia. Among other factors, hypoxia is the most common cause of therapy-resistance CSCs, which activates the hypoxia inducible factor signaling pathway and triggers cellular processes that can lead to a better survival and expansion of CSCs^[26]. The presence of hypoxia in the tumor tissue or its decrease by reoxygenation in the course of chemo/radiotherapy could be correlated with an accelerated repopulation of CSCs with therapy-resistance phenotype^[27].

There are several critical proliferation-promoting and survival-inducing pathways triggering the maintenance and survival of CSCs. The canonical Wnt pathway, which is central signaling pathway for stem cell maintenance and development, is constitutively active in breast cancer, colorectal cancer, myeloid leukemia, lung cancer and skin cancer^[28,29]. Hedgehog Signaling (HH), which has three different homologues desert Hedgehog, Indian

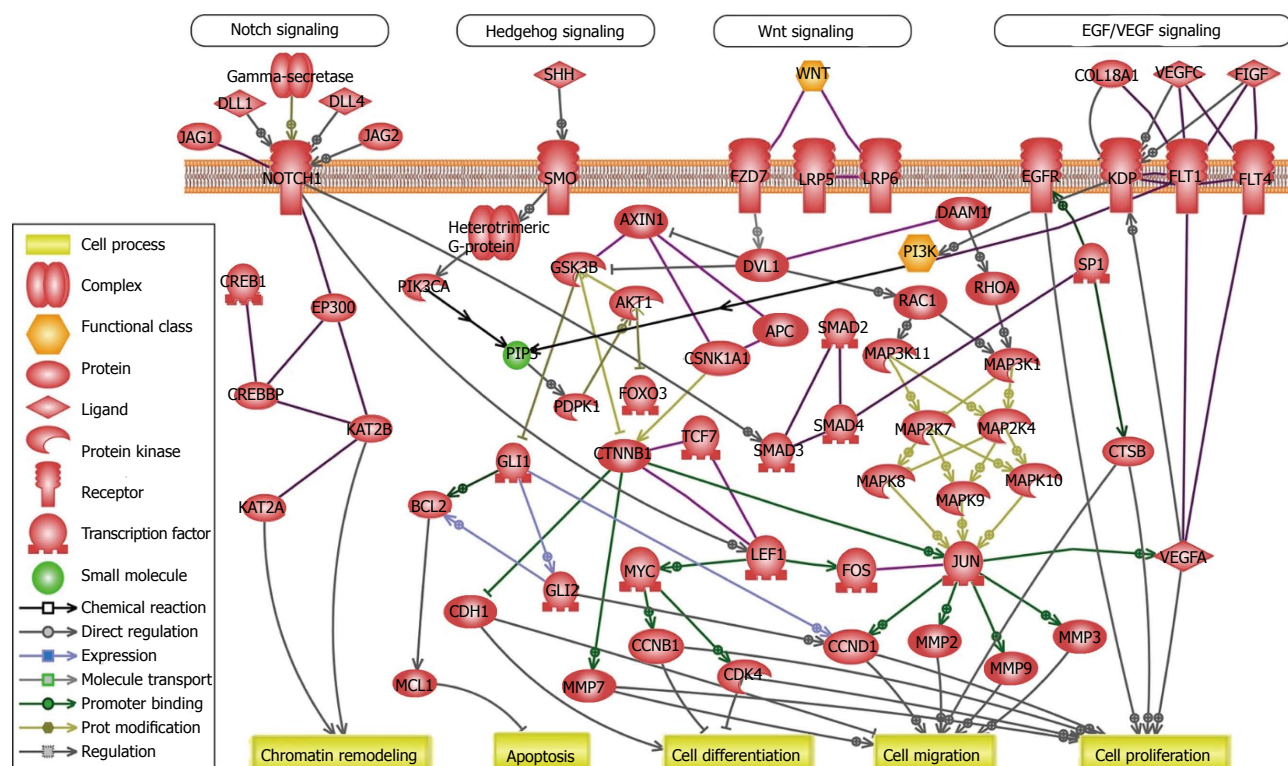


Figure 2 Crosstalk between cancer and cancer stem cell-related pathways. Predicted crosstalk among Wnt signaling, Notch pathway, Hedgehog signaling and other cancer-related pathways like EGF/VEGF signaling in CSCs and cancer. Gene networks and canonical pathways were assessed using the Ariadne Genomics Pathway Studio® program and database (Elsevier). EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor; WNT: Wnt signaling pathways; PI3K: Phosphoinositide 3-kinase; PIP3: Phosphatidylinositol 3,4,5 trisphosphate; CSC: Cancer stem cell; SHH: Sonic Hedgehog.

Hedgehog and Sonic Hedgehog is essential in a variety of molecular and cellular processes during tissue homeostasis, development or embryogenesis. Aberrant HH activation which regulates the CSC's maintenance and potential proliferation, is reported in different cancers including acute myeloid leukemia (AML), breast cancer, chronic myeloid leukemia (CML), glioblastoma, lung carcinoma, myeloma, pancreatic adenocarcinoma^[7,30,31]. Canonical Notch signaling is the other conserved signaling pathway in tissue homeostasis and development. Activation of Notch signaling upon binding of the extracellular ligands, regulates the expression of target genes involving in CSC self-renewal such as Myc, Nanog, Oct-4, and Sox2^[32]. Abnormal Notch activation plays a critical role in breast cancer, myeloid leukemia (AML and CML), glioblastoma, lung cancer and pancreatic cancer^[7,32,33]. Phosphoinositide-3-kinase/protein kinase B, canonical and non-canonical nuclear factor- κ B (NF- κ B), stromal-derived factor-1 α /CXCR4, ErbB signaling and hedgehog/glioma-associated oncogene are other critical pathways that regulates CSC-related maintenance and proliferation^[34-38]. The majority of cancer and CSC-related pathways do not act as isolated units but rather often interact with other pathways as a linked biological network. The predicted crosstalk among Wnt signaling, Notch pathway, Hedgehog signaling and other pathways like EGF/VEGF signaling is illustrated in the Figure 2.

Therefore, therapies that target CSCs could be more effective than therapies targeting a general reduction in tumor mass. Thus, it can be postulated that the

efficacy of the chemo/radiotherapy to eradicate CSCs, can be enhanced by a combination therapy with drugs specifically targeting CSCs (Figure 1).

METHODS FOR SCREENING OF CSCs

Over the past decade, different CSC markers were identified in a wide range of hematopoietic malignancies and solid tumors^[39,40]. A widely used method for characterizing CSC-related markers is multiparameter flow cytometry. This method, which is originally developed for the analysis of blood cells and hematopoietic stem cells, offers the possibility to detect CSCs by means of specific surface markers that are stained with fluorescence-coupled antibodies. Frequently, the expression of CD133 or CD44 alone or in combination with further markers such as CD20, CD24, CD90 or α 2- β 1-integrin is used as a CSC-specific marker (Figure 3). Functional detection of CSC is also possible and is based on the increased expression of detoxification enzyme aldehyde dehydrogenase 1 (ALDH1) or the high activity of multidrug resistance transport proteins. These CSC-specific staining methods allow the isolation of single CSCs for further molecular characterization using single cell based molecular approaches (Figure 3). However, identified markers are not always reliable and none of the reported markers solely identify CSCs, therefore need to be used with caution (Table 1).

For example, inter- or intra-tumor heterogeneity

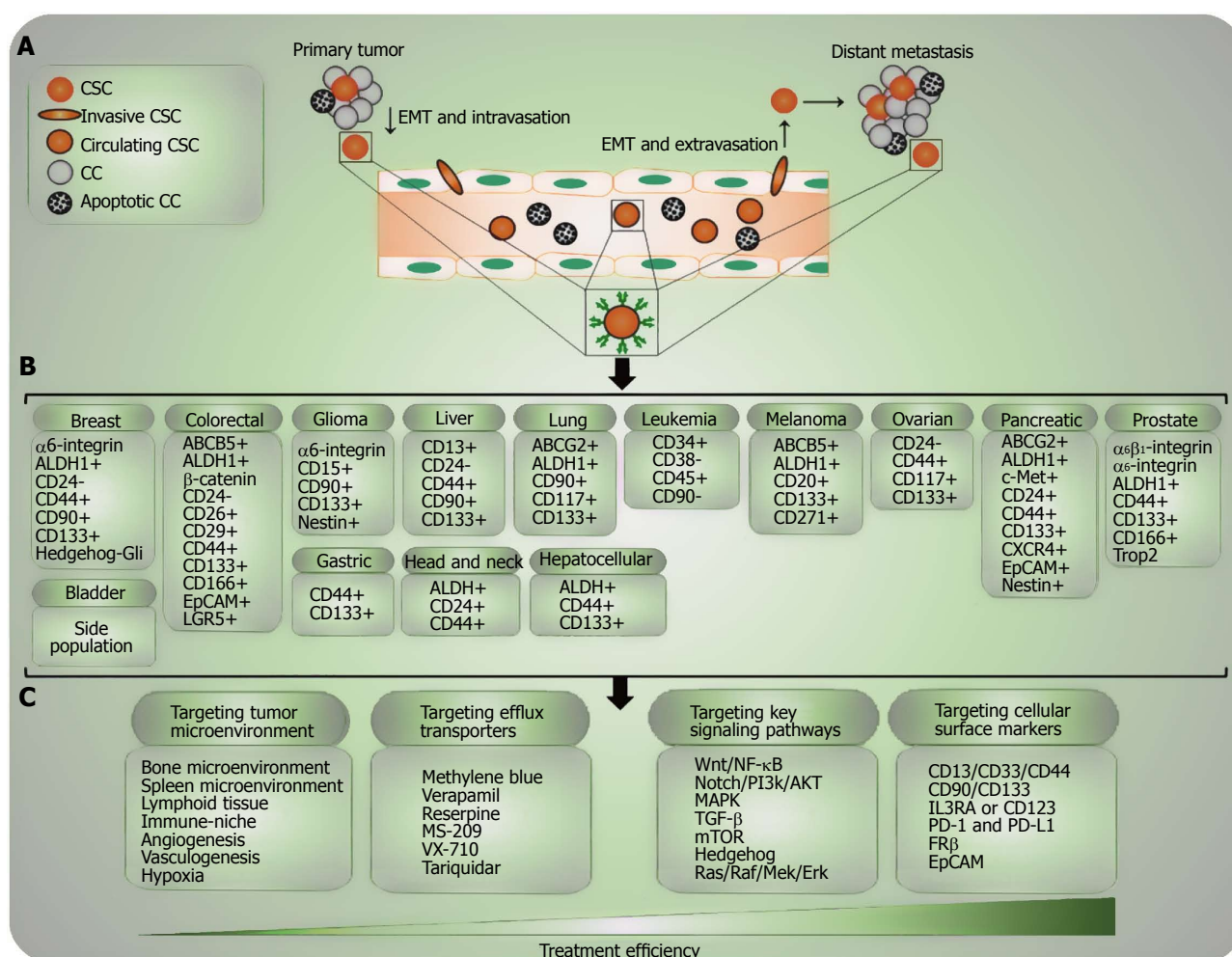


Figure 3 Tracing and targeting cancer stem cells. A: The complex process of distant metastasis including invasion of the tumor microenvironment, EMT, shedding of CSCs into the blood stream (intravasation), MET and invasion of circulating CTCs to the other tissues (extravasation). Only circulating CSCs are able to survive in the circulating blood, escape from immune-surveillance and home to secondary organs; B: The list of known compilation CSC-related molecular markers for different solid tumors and hematopoietic malignancies. The level of specificity of these markers differs per each type of tumor. Markers are ordered alphabetically and not according to their sensitivity or specificity; C: Four important approaches of CSC-targeted therapy. CSC: Cancer stem cell; CC: Cancer cell; NC: Normal cell; EMT: Epithelial-mesenchymal transition; MET: Mesenchymal-epithelial transition; PI3K: Phosphoinositide 3-kinase; MAPK: Mitogen-activated protein kinase; TGF: Transforming growth factor; mTOR: Mechanistic target of rapamycin; RAS: Ras-activated signaling; PD-1: Programmed death 1; PD-L1: Programmed death-ligand 1; EpCAM: Epithelial cell adhesion molecule.

may completely render CSC markers inapt. Such tumor heterogeneity can be the result of different genetically distinct clones within the tumor due to having various genetic lesions or dysregulation of markers via pathologic epigenetic regulations^[16,37-43]. For example, CD133 marker is frequently inactivated due to the DNA methylation and therefore often inadequate^[44]. Inactivation of specific markers due to any escape mechanism in a particular clone may render these CSCs undetectable in the absence of other distinct markers.

While high-throughput genetic screening studies provide essential information about genes which are associated with a particular phenotype, molecular pharmacology can play an important role in development of a specific molecular therapy. Low molecular weight substances ("small molecules") show a higher penetrance in cell-based screening methods. Therefore, small molecules are one of the most frequently used therapeutic

agents. The screening of large substance banks has identified many valuable compounds that can be used to modulate biological systems in cancer cells^[45]. In order to systematically identify the genes that regulate the death and differentiation of CSCs, high-throughput screenings of RNA interference (RNAi) or chemical substance libraries are carried out using different approaches. The readout of such screen approaches can be survival analysis, reporter assays, luminescence or fluorescence-based analyzes of particular genes or pathways and imaging methods, in which several cellular properties can be examined on a single cell level.

Since CSCs only make up a small fraction in the entire tumor cell pool (Figure 1), appropriate enrichment methods must be applied. Gupta *et al*^[46] enriched CD44^{hi}/CD24^{lo} cells within the CSC population of mammary carcinoma cell lines by inducing the EMT. After treatment with inhibitors, the survival of the enriched and

Table 1 Hallmarks of using cancer stem cell-related markers

Problems	Potential solutions
CSC-related markers may not be specific by their own for a certain type of tumor	Combined used of different markers may be the solution
Some of CSC-related markers may be down-regulated or suppressed in a given tumor due to different genetic or epigenetic regulatory mechanisms	Using of distinct markers or a combination them
Splice variants of some CSC-related markers may render detection difficult	The exact splice variant should be considered for the detection
Markers can be detected using one method (e.g., FACS), but not with other methods (e.g., immunohistochemistry)	Stringent selection of related markers might be required
Different tumors have clonal variation and heterogeneous cell population. Less malignant clones may harbor CSCs that express different markers. Therefore, CSC-related markers may be differentially regulated within different clone or be completely missed	Using more specific and sensitive methods, isolate more enriched CSC populations
Many of reported CSC-related markers are not validated, since they derived from cell-line or mouse model studies	Markers should be validated in xenotransplants or primary human materials

CSC: Cancer stem cell; FACS: Fluorescence-activated cell sorting.

the non-selected cell population was investigated using a luminescence-based reporter assay. This study was able to identify salinomycin as a selective inhibitor of the CSC population in breast carcinoma^[46].

Recent advances in computer-based image analysis have enabled rapid achievements in the development of image-based high-throughput analysis approaches. The direct visualization of cellular features and biological processes allows a more comprehensive measurement of responses to interferences. Xia *et al*^[47] have developed a novel fluorescence imaging method to identify cancer cells with CSC properties through their increased ability to deliver fluorescent dyes *via* dedicated molecular transporters. Based on this method, a library of active substances was examined for their effect in CSCs. It was possible to identify substances that selectively inhibit the molecular transporters^[47].

A further high-throughput method has recently been developed to characterize the biochemical and biophysical environmental conditions of CSCs. Microarray glass slides with over 2000 test chambers can be used to cultivate stem cells in different cell densities in a hydrogel of polyethylene glycol, to which different biological molecules have been coupled by robot technology^[48]. Using the microscopic imaging, cell proliferation, morphology and differentiation can be monitored at a single cell level. This method as a platform for the investigation of individual stem cells in a microfluid culture system with simultaneous live-cell microscopy, represents an important step towards the miniaturization of the cellular processes as a high-throughput screening approach^[49].

TARGETING CSCs

Targeting tumor microenvironment

The heterogeneous tumor microenvironment or cancer cell-niche, provides different self-protection mechanisms which enables a dynamic interaction with surrounding cells including immune cells, cytokines and chemokines to regulate proliferation, maintenance and self-renewal of CSCs. CSCs can represent more autonomous regulatory

characterization in an independent manner^[13]. Less malignant tumors may have more demand on the stem cell-niche but upon cancer progress this dynamic interplay might be weakened or even diminished^[14]. It is known that dormant cancer cells *via* reducing their immunogenicity, can escape the immune surveillance^[50]. Therefore, targeting CSC microenvironment may stimulate the host antitumor responses^[51]. Strategies to hit the tumor-promoting inflammation are under investigation. Production of prostaglandin E2 (PGE2) by tumor cells in breast cancer, colorectal cancer and melanoma has a key role in the escape phase as it suppresses immunity and induces inflammation^[52]. Therefore, the use of antagonists of PGE2 receptor (PTGER4) has proven successful in blocking immuno-suppression and preventing cancer metastases^[53].

Targeting efflux transporters

Membrane efflux transporters, which are mainly located in blood-brain barrier, hepatocytes, intestinal cells or kidney proximal tubules, play important roles in drug metabolism, availability, and toxicity of drugs in human body^[54]. Several studies indicate that transporter-mediated drug disposition plays an important role in mediating chemo-sensitivity and -resistance of cancer cells and CSCs^[55]. The interaction between efflux transporters and chemotherapeutic drugs on cancer cells is significantly linked to the efficacy of cancer therapy. Two major superfamilies of efflux transporters are the ATP-binding cassette (ABC) transporters [ABCB1 (MDR1), ABCC1 (MRP1), ABCC2 (MRP2) and ABCG2 (BCRP)] and the solute carrier (SLC) transporters [SLC19A1 (RFC1) and SLC01B1 (SLC21A6)]. Therefore, targeting efflux transporters within cancer therapy combined with routine therapies could significantly increase the eradication rate of resistant cancer cells^[56].

Targeting key signaling pathways

The CSC phenotype depends on various cellular signals, which are triggered by the underlying genetic lesions and by the support of the stem cell niche. Some of

these signals have already been identified; the most disease cussed signaling pathways are the classic Wnt- β -catenin, Notch and Sonic Hedgehog signaling^[57-59]. For these three pathways, pharmacological inhibitors have been developed which are now undergoing clinical trials in many independent studies^[60]. However, the clinical effect is largely depending on the tumor type and not all three pathways are equally important in all types of tumors. It has been shown that, although some signaling pathways are highly tumor-promoting in a certain type of cancers (which makes it a suitable therapeutic target), they might react as tumor suppressive in another tumor type; therefore, their inhibition may become dangerous (e.g., Notch-1 has been identified as a tumor suppressor in urinary bladder carcinoma)^[61]. High Wnt pathway activity marks colon or leukemia CSCs and is required for stemness signature as a prognostic marker^[6,7,62]. In addition, Wnt activity is associated with the CSC markers CD133, CD44 and LGR5 in colon cancer^[63] whereas Hedgehog activity is linked to ABC transporter expression in esophageal and prostate cancer^[17,64] TGF- β signaling *via* the family members Nodal and Activin is attributed to pancreatic CSCs^[65]. The effect of Hedgehog inhibitors is actually the most evident in the basal cell carcinoma. In addition, inhibition of Hedgehog pathway blocks stemness in breast CSCs, whereas its activation enhances self-renewal^[66]. It is also necessary to distinguish whether those signaling pathway has been activated within CSCs only because of harbored genetic lesions^[67]. If only the CSCs are targeted, it is hardly possible to expect a dramatic tumor shrinkage as in classical successful chemo/radiotherapy; rather, this would be a disease stabilization and a slowing of the progression (Figure 1).

Disulfiram was developed as an inhibitor of aldehyde dehydrogenase for the treatment of alcoholism. This inhibition leads to the accumulation of acetaldehyde after alcohol consumption, resulting in a marked nausea that should reduce the probability of further alcohol consumption^[68]. The same enzymatic activity - aldehyde dehydrogenase - is, however, a component of the self-protection of the CSCs, and thus disulfiram was used for the elimination of CSCs. Thioridazine is an inhibitor of dopamine receptors, and is a standard medication for mental disorders such as schizophrenia. Its rational use in tumor therapy is based on the finding that CSCs in several types of tumors (e.g., AML, breast carcinoma, glioblastoma), in contrast to the corresponding normal tissue stem cells, upregulate the expression of dopamine receptors^[69].

Nicosamide was identified that specifically targeted Wnt- β -catenin signaling pathways^[70]. Interestingly, nicosamide is known as an antiparasitic and inhibitor of oxidative phosphorylation, which has been used in human medicine for almost 50 years^[71]. However, what has emerged is that these two antiparasitics are inhibitors of numerous other signaling pathways. Nicosamide inhibits not only the Wnt- β -catenin signaling pathway, but also the Notch, PI3'K-Akt - mTOR, STAT-3 and

NF κ B signaling pathways, which are essential for tumor stem cells^[72]. Salinomycin was similarly described as an inhibitor of ABC efflux pumps and the Wnt- β -catenin signaling pathway^[73]. Not enough, analogous effects have been discovered for disulfiram and thioridazine. Disulfiram has not only proved to be an effective inhibitor of aldehyde dehydrogenase, but also polo-like kinase 1 and O6-methylguanine methyltransferase as well as NF κ B^[74,75].

The advantages of identifying such new indications for old drugs are obvious. These drugs have long been out of patent protection and their use should therefore be much cheaper than for newly developed drugs, which is an important aspect in the current discussion on costs of tumor treatment. In addition, they have already undergone clinical trials, their potential toxicity, side effects, pharmacokinetics, contraindications, and possible drug-drug interactions are known. Therefore, their use in tumor therapy should be relatively easy. Perhaps the best opportunity to see how the effects of tumor stem cell-targeted therapy can be demonstrated is in the area of combination therapy (Figure 1). Tumor stem cell-directed drugs should be able to prolong the efficacy of cytotoxic therapy and reduce recurrence risk^[76,77]. On the other hand, combined administration has significantly greater chances of total elimination of all tumor cells. Taken together, there are many possibilities for therapeutic treatment for the elimination of tumor stem cells, both from the group of newly developed inhibitors of some stem cell-specific signaling pathways as well as for some old drugs that can find a new application in tumor therapy.

Targeting cellular surface markers (tumor immunotherapy and cancer vaccination)

Many types of normal cells like immune cells infiltrate tumors. Over the last years, immune infiltration has become a central focus in cancer research^[50]. It is increasingly recognized that cancer cells and CSCs need to escape immune recognition. IL-6/JAK/STAT3 signaling an important pathway in many solid tumors. Anti-IL-6 mAb siltux-imab was tested on various cancer types, which was not able to provide promising outcome to improve overall survival of patients with multiple myeloma according to a recent Phase II clinical trial on patients^[78]. While, checkpoint blockade antibodies such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) or programmed death-ligand 1 (PD-1/PD-L1) like ipilimumab or nivolumab could provide marked clinical benefits for lung adenocarcinoma, melanoma or Hodgkin lymphomas^[79,80]. These agents can boost the immune system and display clinical benefits for a fraction of patients^[50].

Many tumors cells including CSCs, alter the expression of their genes or down-modulate of antigen processing and presentation to build an immuno-suppressive microenvironment that creates physical or chemical barriers against immune cells^[81]. Indeed, CSCs by low express of MHC-I, and over expressing of IL-4 are escaping from cytotoxic T lymphocytes^[82].

Boosting T-cell response can be a promising approach to eradicate CSCs. This can be achieved by boosting neo-antigens within CSCs, considered as tumor vaccination. Adoptive transfer of CSC-specific T-cells into tumor-bearing mice could show a success^[83]. In addition, genetically modified T cells to express chimeric antigen receptors (CAR T-cells) upon adaptive transfer could provide remarkable benefit for patients suffering from different solid tumors or leukemia^[84]. Therefore, the major goal of immunotherapy is to thwart these barriers in order to enhance pre-existing or elicit a new immune response against cancer.

CONCLUSION

Because of the CSCs' ability to therapy resistance and initiate a recurrence after therapy, cancer stem cell is an important therapeutic target. Future research is essential to elucidate how CSCs dictate metastasis, therapy-resistance or immune-scape signature. However, without having reliable markers it will be a challenging pursuit. An exact molecular characterization of this small subpopulation in the tumor tissue requires the development of specific CSC markers and suitable enrichment methods. Particularly from innovative high-throughput screening technologies, we can expect valuable insights regarding suitable CSC-associated biomarkers and new therapeutic approaches to target CSCs. This could be an important step towards individualized cancer therapy.

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Basic Study

Aldehyde dehydrogenase activity helps identify a subpopulation of murine adipose-derived stem cells with enhanced adipogenic and osteogenic differentiation potential

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Abstract

AIM

To identify and characterize functionally distinct subpopulation of adipose-derived stem cells (ADSCs).

METHODS

ADSCs cultured from mouse subcutaneous adipose tissue were sorted fluorescence-activated cell sorter based on aldehyde dehydrogenase (ALDH) activity, a widely used stem cell marker. Differentiation potentials were analyzed by utilizing immunocytofluorescence and its quantitative analysis.

RESULTS

Approximately 15% of bulk ADSCs showed high ALDH activity in flow cytometric analysis. Although significant difference was not seen in proliferation capacity, the adipogenic and osteogenic differentiation capacity was higher in ALDH^{hi} subpopulations than in ALDH^{lo}. Gene set enrichment analysis revealed that ribosome-related gene sets were enriched in the ALDH^{hi} subpopulation.

CONCLUSION

High ALDH activity is a useful marker for identifying functionally different subpopulations in murine ADSCs. Additionally, we suggested the importance of ribosome for differentiation of ADSCs by gene set enrichment analysis.

Key words: Adipose-derived stem/stromal cell; Aldehyde dehydrogenase activity; Flow cytometry; Subpopulation; Ribosome

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Core tip: Aldehyde dehydrogenase (ALDH) activity is widely used as a stem cell marker in several types of normal or malignant tissues. However, there was no report of ALDH activity in murine adipose-derived stem cells (ADSCs). Here, our study demonstrated a subpopulation defined by high ALDH activity within murine ADSCs. The subpopulation with high ALDH activity (ALDH^{hi}) showed enhanced differentiation potentials into adipocyte and osteocyte. Furthermore, gene set enrichment analysis revealed that ribosome-related gene sets were enriched in ALDH^{hi} of murine ADSCs. We showed relationship between ALDH^{hi} and ribosome biosynthesis, providing a novel insight of mesenchymal stem cell biology.

Itoh H, Nishikawa S, Haraguchi T, Arikawa Y, Eto S, Hiyama M, Iseri T, Itoh Y, Nakaichi M, Sakai Y, Tani K, Taura Y, Itamoto K. Aldehyde dehydrogenase activity helps identify a subpopulation of murine adipose-derived stem cells with enhanced adipogenic and osteogenic differentiation potential. *World J Stem Cells* 2017; 9(10): 179-186 Available from: URL: <http://www.wjgnet.com/1948-0210/>

INTRODUCTION

Stem cells can self-renew and differentiate into specialized cells of various tissues^[1]. Therefore, these cells, for example, embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), hematopoietic stem cells, and mesenchymal stem cells (MSCs), have been the object of basic research and clinical applications. Among these types of stem cells, MSCs, as represented by adipose-derived stem/stromal cells (ADSCs) and bone marrow-derived stem/stromal cells (BMSCs), have been recognized as useful material for cell-based therapy^[2]. MSCs have been isolated from various tissues, including adipose tissue, the bone marrow, peripheral blood, cord blood, the liver, dental pulp, and fetal tissue; of these, adipose tissue is one of the most abundant source of MSCs^[3]. ADSCs possess multipotency and have the potential to differentiate into cell types such as adipocytes, osteocytes, chondrocytes, neurons, vascular endothelial cells, cardiomyocytes, myoblasts, and islet β -cells under appropriate conditions^[4].

The researches have suggested that ADSCs are heterogeneous and comprise phenotypically and/or functionally different subpopulations^[5-7]. For example, the cluster of differentiation (CD)73⁺ subpopulation of murine ADSCs possesses increased potential for cardiomyocyte differentiation^[6]. The CD90⁺ subpopulation of murine ADSCs has higher tube-forming ability than the CD90⁻ subpopulation, which has high adipogenic potential^[8]. The CD90⁺ subpopulation also exhibits higher efficiency of iPSC induction than the CD90⁻ subpopulation^[5]. Human ADSCs also include the CD105^{lo} subpopulation, which has high osteogenic potential^[7]. Some studies have identified different subpopulations in ADSCs on the basis of surface antigen markers^[5-7]. However, it is unclear how these markers (e.g., CD90 and CD105) are functionally related to cell differentiation.

In mice, aldehyde dehydrogenase (ALDH) is a superfamily comprising 20 intracellular enzymes and is responsible for the oxidization of various aldehydes^[9]. High ALDH activity has been shown in normal hematopoietic stem cells, neural stem cells, and cancer stem cells in various types of neoplastic diseases^[10]. Therefore, high ALDH activity is considered to be a common marker for normal and malignant stem cells. In human ADSCs, however, only one study has been performed on the ALDH^{hi} subpopulation, whose significance in differentiation potential is unclear^[11]. Moreover, to our knowledge, the existence of the ALDH^{hi} subpopulation within murine ADSCs has not yet been reported.

In the current study, the ALDH^{hi} and ALDH^{lo} subpopulations of murine ADSCs were sorted using flow cytometry. The differentiation potential and proliferation of the sorted ALDH^{hi} and ALDH^{lo} subpopulations were analyzed. Furthermore, we analyzed the transcriptional profiles of the ALDH^{hi} and ALDH^{lo} subpopulations by utilizing gene set enrichment analysis (GSEA).

MATERIALS AND METHODS

Animals and ADSC isolation

C57BL/6J mice were purchased from Kyudo Co., Ltd (Saga, Japan). All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the institutional guidelines of Yamaguchi University. The animal experiments were approved by the institutional animal experiment ethics committee of Yamaguchi University.

Murine ADSCs were isolated from twenty of C57BL/6J female mice of 4- to 6-wk-old, as previously described^[8]. Briefly, the subcutaneous adipose tissue was resected, washed with Dulbecco's phosphate-buffered saline (DPBS; Wako, Osaka, Japan), and cut into small pieces. The adipose tissue pieces were digested in high glucose Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) containing 1.0 mg/mL collagenase type I (Sigma-Aldrich, St. Louis, MO, United States), 10% fetal bovine serum (FBS; Sigma-Aldrich), and antibiotic-antimycotic agents (PSM; penicillin: 100 U/mL, streptomycin: 100 µg/mL, and amphotericin B: 0.25 µg/mL, final concentrations; Nacalai Tesque, Kyoto, Japan), using a shaking incubator at 37.5 °C and 250 rpm for 1 h. The digested tissue was filtered through a sterile ø100-µm nylon mesh (EASystrainer, 100 µm; Greiner Bio-One Japan, Tokyo, Japan), followed by centrifugation at 400 × *g* for 5 min in DPBS supplemented with 1% FBS and 1 mmol/L EDTA-3Na (Wako, Osaka, Japan). The pellet was resuspended in DMEM supplemented with 10% FBS and antibacterial/antimycotic agent and was cultured at 37.0 °C in a 5% CO₂ atmosphere, using ø10 cm dish (Corning, NY, United States). When the cultures reached 80%-90% confluence, the ADSCs were dissociated from the dish by using Accutase solution (Innovative Cell Technologies, San Diego, United States), and seeded into new dishes.

Flow cytometry analysis

Adherent ADSCs from passage 4 were dissociated using Accutase solution; 1 × 10⁶ cells were resuspended and incubated for 5 min on ice with 2 µL of anti-mouse CD16/32 rat monoclonal antibody (BioLegend, San Diego, CA, United States). Cells were stained with 1 µL viability probe (Zombie NIR, Biolegend) for 20 min at room temperature to stain dead cells. ALDH activity was assessed by utilizing the ALDEFLUOR kit (Stemcell Technologies, Vancouver, Canada) according to the manufacturer's instructions. Briefly, 1 × 10⁶ cells were resuspended in 1 mL assay buffer and 5 µL ALDEFLUOR reagent was added after thorough mixing; then, 0.5 mL of the cell suspension was transferred to a new tube with 5 µL diethylaminobenzaldehyde (DEAB) reagent (ALDH inhibitor) for negative control of ALDH activity. Flow cytometric analysis and cell sorting were performed using Accuri C6 (BD Bioscience, San Jose, CA, United States) and the SH800 cell sorter (Sony, Tokyo, Japan). Flow cytometric data were analyzed with the FlowJo (Tree Star,

Ashland, OR, United States) software.

Measurement of proliferation potential

To assess the viability of the ADSC subpopulations, we used a cell WST-8 assay (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, sorted ALDH^{hi} or ALDH^{lo} murine ADSCs were seeded in 96-well plates at a density of 3 × 10³ cells/well. After 12, 24, 48, and 72 h, 100 µL fresh medium containing 10 µL CCK-8 solution was added to each well, followed by incubation at 37 °C for 1 h. The absorbance of each well at 450 nm was measured using an Epoch microplate spectrophotometer (BioTek Instruments, Winooski, VT, United States). Six replicates were prepared for each group.

Cell differentiation and immunofluorescence staining

The adipogenic and osteogenic differentiations of ADSCs were characterized using a Mouse Mesenchymal Stem Cell Functional Identification Kit (R and D Systems, Minneapolis, MN, United States) according to the manufacturer's instructions. Briefly, for adipogenic differentiation, cells (3 × 10³/well) were cultured at 37 °C in a 5% CO₂ atmosphere in a 96-well plate in 100 µL adipogenic differentiation medium composed of α-minimal essential medium (αMEM) supplemented with 10% FBS, 1% PSM, L-glutamine, and 50 µL adipogenic supplement containing hydrocortisone, isobutylmethylxanthine, and indomethacin for 15 d in 37 °C and a 5% CO₂ atmosphere.

For osteogenic differentiation, cells were cultured in osteogenic differentiation medium composed of 5 mL α-MEM basal medium and 250 µL osteogenic supplement containing ascorbate-phosphate, β-glycerolphosphate, and recombinant human bone morphogenetic protein-2 for 15 d in 37 °C and a 5% CO₂ atmosphere. The medium was replaced every 2-3 d.

To assess adipogenic and osteogenic differentiation by immunocytochemistry, cultured cells were fixed in 4% paraformaldehyde phosphate buffer solution (Wako, Osaka, Japan) for 20 min. After the cells were washed with DPBS, they were permeabilized and blocked with DPBS supplemented with 0.3% Triton X-100 (Sigma-Aldrich), and 10% FBS for 45 min. The cells were subsequently incubated for 1 h in DPBS containing 10 µg/mL goat anti-mouse fatty acid binding protein (FABP) 4 polyclonal antibody to label adipocytes or were incubated with 10 µg/mL goat anti-mouse osteopontin polyclonal antibody to label osteocytes. They were then washed with DPBS and incubated for 1 h in DPBS containing phycoerythrin (PE)-conjugated rabbit anti-goat IgG antibody [rabbit F(ab')₂ anti-goat IgG-H and L (PE), pre-adsorbed, Abcam, Cambridge, United Kingdom]. Nuclei were stained with 5 µg/mL Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan). Photographs were obtained and analyzed using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan) and its analysis software.

Gene expression array analysis and GSEA

Gene expression array analysis and GSEA were performed

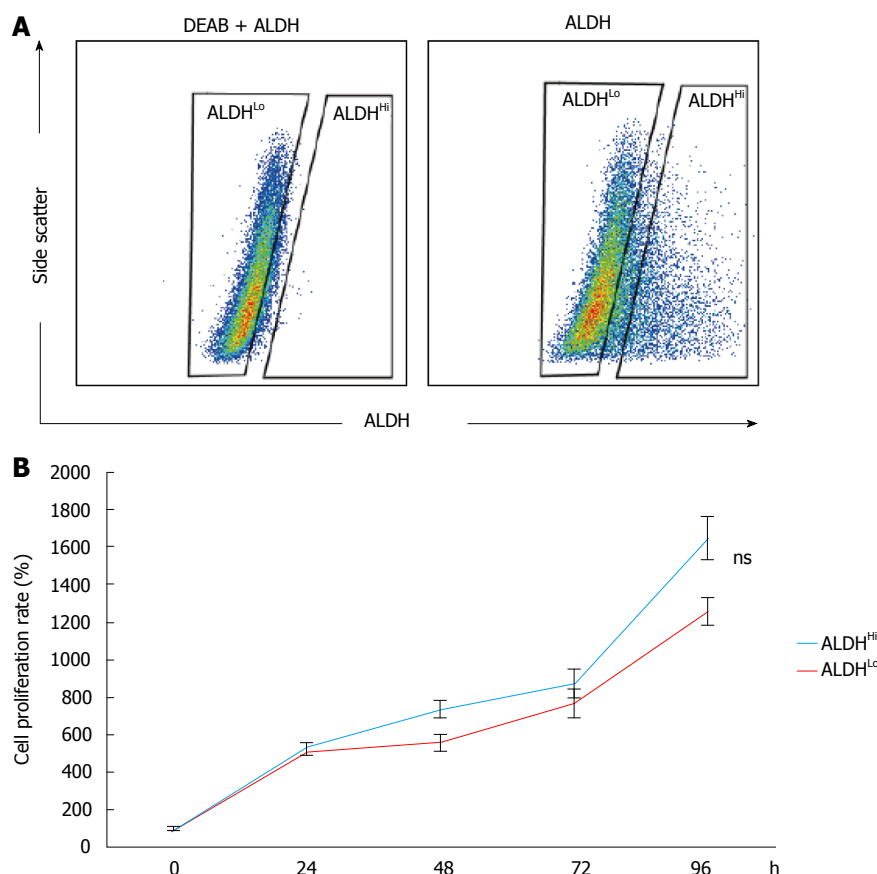


Figure 1 Detection of aldehyde dehydrogenase-positive subpopulations of murine adipose-derived stem cells and evaluation of proliferation rates. A: Flow cytometric analysis of murine ADSCs. Baseline fluorescence was established by adding the ALDH inhibitor diethylaminobenzaldehyde; B: Cell proliferation rates were not significantly different between the ALDH^{Hi} and ALDH^{Lo} subpopulations. Values have been expressed in terms of mean \pm SE ($n = 5$). ns: Not significant; ALDH: Aldehyde dehydrogenase; ADSCs: Adipose-derived stem cells; DEAB: Diethylaminobenzaldehyde.

on the published gene expression profile of C57BL/6 mice divided by ALDH^{Hi} and ALDH^{Lo} subpopulations of ADSCs. About 3×10^6 cells from each subpopulation were lysed and total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Cyanine-3 (Cy3)-labeled cRNA was prepared from 0.1 μ g total RNA by using the Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, United States) according to the manufacturer's instructions; this was followed by RNeasy column purification (Qiagen). Dye incorporation and cRNA yield were checked with the NanoDrop ND-2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). Cy3-labelled cRNA (0.6 μ g) was fragmented at 60 °C for 30 min in a reaction volume of 25 μ L containing 1 \times Agilent fragmentation buffer and 2 \times Agilent blocking agent following the manufacturer's instructions. On completion of the fragmentation reaction, 25 μ L of 2 \times Agilent hybridization buffer was added to the fragmentation mixture and hybridized to SurePrint G3 Mouse GE 8 \times 60 K Ver1.0 (Agilent Technologies) for 17 h at 65 °C in a rotating Agilent hybridization oven. After hybridization, the microarrays were washed for 1 min at room temperature with GE Wash Buffer 1 (Agilent Technologies) and 1 min with 37 °C GE Wash buffer 2 (Agilent Technologies). The slides were scanned immediately after washing on the Agilent SureScan

Microarray Scanner (G2600D), using one color scan setting for 8 \times 60 k array slides (scan area, 61 \times 21.6 mm; scan resolution, 3 μ m; dye channel set for Green PMT was set to 100%). The scanned images were analyzed with Feature Extraction Software 11.5.1.1 (Agilent Technologies), using default parameters to obtain the subtracted background and spatially detrended Processed Signal intensities.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla, CA, United States). The results have been expressed in terms of mean \pm SE. Comparisons of two groups were performed with the independent *t*-test. Multiple comparisons were performed with one-way analysis of variance. Data were considered statistically significant when the *P* value was ≤ 0.05 .

RESULTS

ALDH activity of murine ADSCs

To identify the subpopulation defined by ALDH activity in murine ADSCs, single-cell suspensions of cultured murine ADSCs were stained using the ALDEFLUOR kit and analyzed with flow cytometry. A small subpopulation

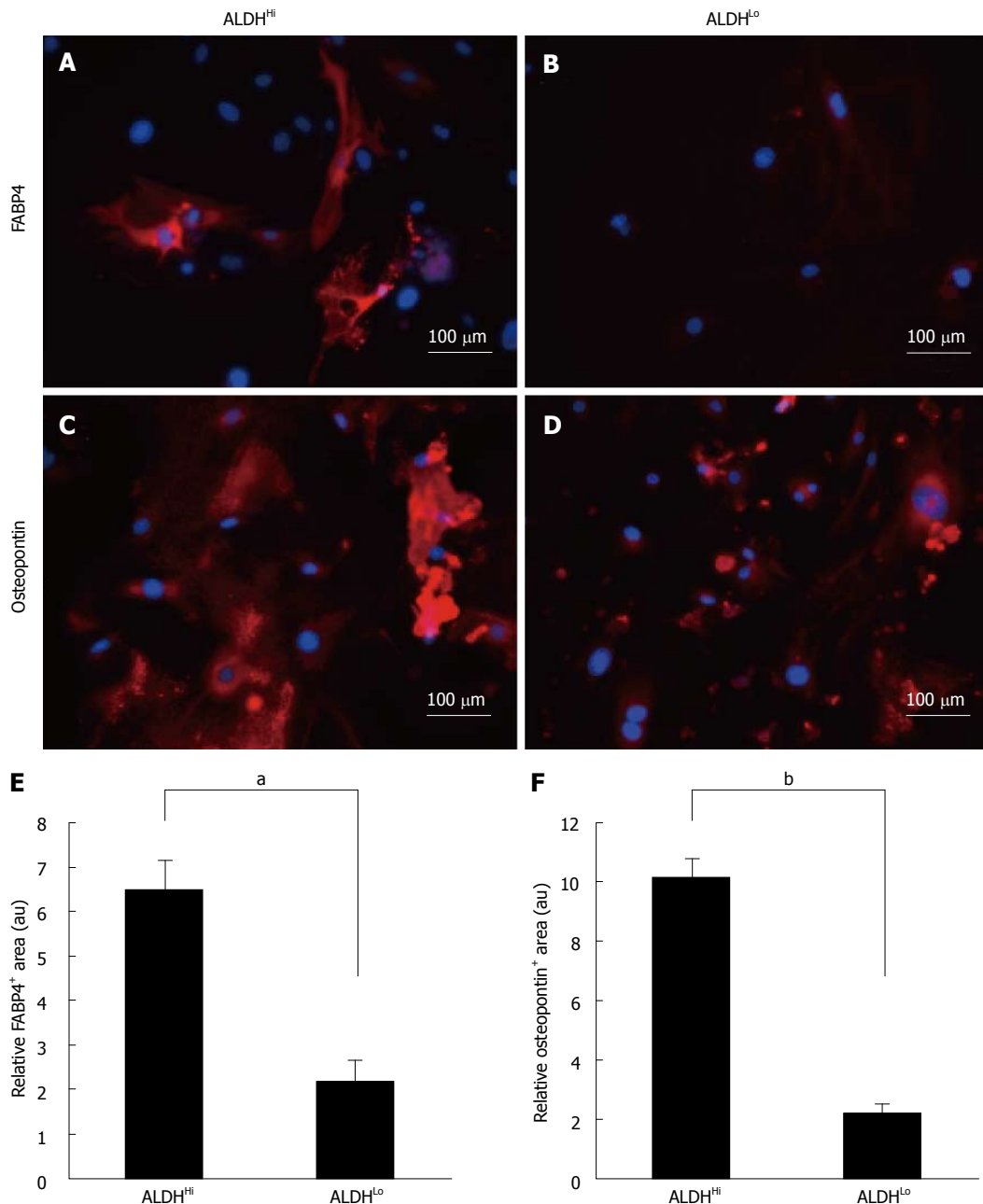


Figure 2 Marker analysis of differentiation potentials. Differentiation potential of ALDH^{Hi} (A, C) and ALDH^{Lo} (B, D) subpopulations of ADSCs. FABP4 (A, B) and osteopontin (C, D) expression in ADSCs (red) following adipogenic (A, B) and osteogenic (C, D) differentiation of ALDH^{Hi} and ALDH^{Lo} subpopulations, as determined by immunocytochemistry. The nuclei were stained with Hoechst 33342 (blue). Quantitative analysis of differentiation-marker-positive areas in differentiated ADSCs. FABP4-positive (E) and osteopontin-positive (F) area ratios relative to respective areas of nuclear staining for ALDH^{Hi} and ALDH^{Lo} subpopulations of ADSCs after adipogenic (E) and osteogenic (F) induction. Values have been expressed in terms of mean \pm SE ($n = 5$). ^a $P < 0.05$, ^b $P < 0.0001$, au: Arbitrary unit. ALDH: Aldehyde dehydrogenase; ADSCs: Adipose-derived stem cells; FABP: Fatty acid binding protein.

with distinctively high ALDH activity (ALDH^{Hi} cells) was detected within the bulk populations of ADSCs (Figure 1A). The percentage of ALDH^{Hi} cells was approximately 15% of the bulk murine ADSC population (Figure 1A). However, on adding the ALDH inhibitor N,N-diethylaminobenzaldehyde (DEAB), a distinct ALDH^{Hi} subpopulation was not detected (Figure 1A). To assess the difference in the proliferation potentials of the ALDH^{Hi} and ALDH^{Lo} subpopulations, we measured the proliferation rate of each subpopulation by using the WST assay. The proliferation potential of the ALDH^{Hi} subpopulation of

ADSCs was not significantly different compared to the ALDH^{Lo} subpopulation (Figure 1B).

Cell differentiation to adipocytes and osteocytes

To assess the adipogenic and osteogenic differentiation potential of the two subpopulations, sorted ALDH^{Hi} and ALDH^{Lo} cells were cultured under adipogenic or osteogenic differentiation conditions. After *in vitro* differentiation, immunofluorescence staining for FABP4 (marker of adipocytes) and immunofluorescence staining for osteopontin (marker of osteocytes) were performed

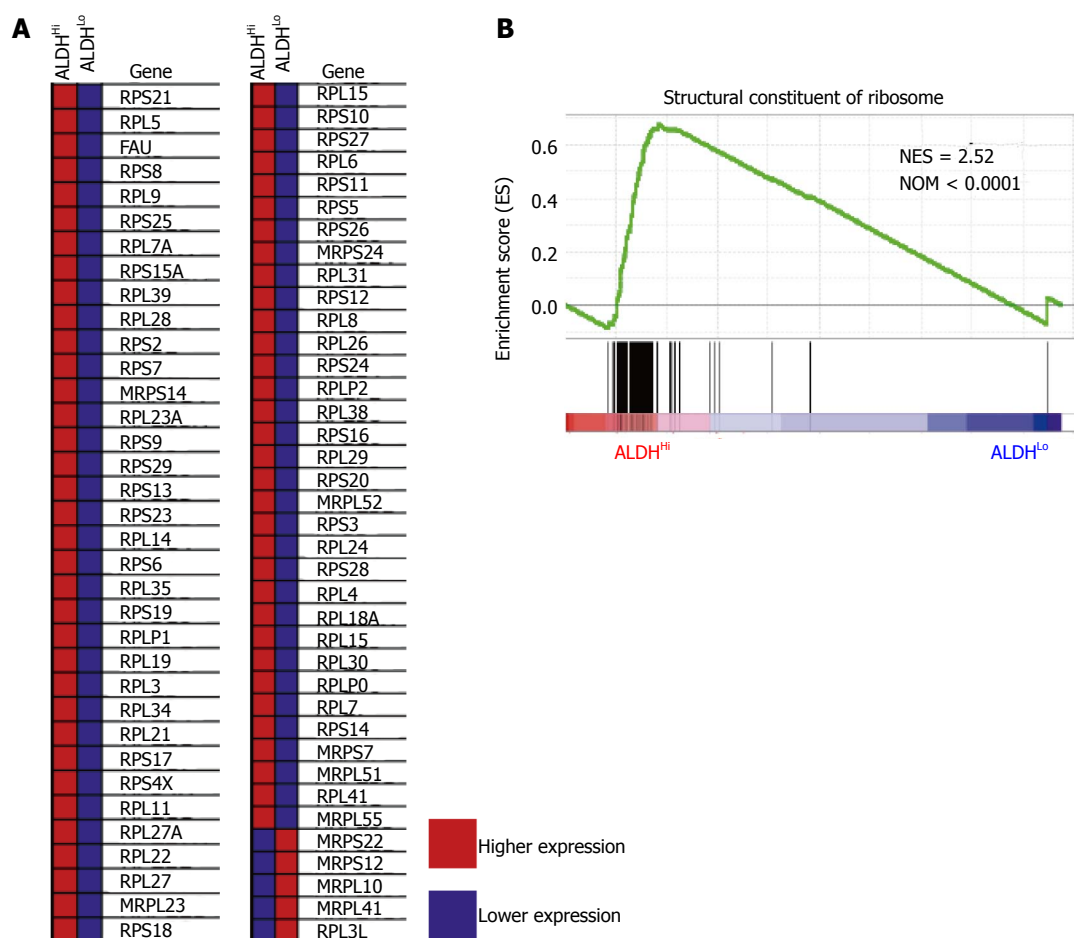


Figure 3 Gene set enrichment analysis of each ALDH^{Hi} and ALDH^{Lo} subpopulation. A: Heat map of enrichment profile of ribosomal protein mRNAs for ALDH^{Hi} and ALDH^{Lo} subpopulations; B: Gene set enrichment analysis of transcription data related to structural constituents of ribosomes for the ALDH^{Hi} and ALDH^{Lo} subpopulations. Normalized enrichment score and nominal *P* values are shown. ALDH: Aldehyde dehydrogenase.

(Figure 2A-D). ADSCs that differentiated into adipocytes appeared as accumulated lipid droplets in the cytosol in each ALDH^{Hi} and ALDH^{Lo} subpopulation (Figure 2A and B). Furthermore, immunofluorescence staining for osteopontin revealed that ADSCs that differentiated into osteocytes appeared as accumulated granules in the cytosol in each ALDH^{Hi} and ALDH^{Lo} subpopulation (Figure 2C and D).

Evaluation of differentiation

Adipogenic and osteogenic differentiation of each ALDH^{Hi} and ALDH^{Lo} subpopulation was quantitatively assessed using the BZ-9000 microscope and its analysis software. Ten visual fields were taken randomly for every 3 wells, and the immunofluorescence-staining positive-areas in 30 visual fields were analyzed. Subsequently, the immunofluorescence-staining-positive area was divided by the Hoechst 33342-positive area for each of the 30 visual fields. The ALDH^{Hi} subpopulation was found to have significantly more adipogenic and osteogenic relative-differentiation-marker-positive areas than the ALDH^{Lo} subpopulation (Figure 2E and F).

GSEA

To identify the sets of gene that were up- or down-

regulated in the ALDH^{Hi} subpopulations, we performed GSEA for the published gene expression profile of C57BL/6 mice divided by the ALDH^{Hi} and ALDH^{Lo} subpopulations of ADSCs. Intriguingly, high gene set enrichment scores were obtained for the structural constituents of ribosomes (Figure 3).

DISCUSSION

MSCs are reported to commonly express CD29, CD73, CD90, and CD105 and to be negative for markers such as CD45 and CD56^[12,13]. There have been many studies on cell surface antigen markers of ADSCs, such as CD34 and CD44^[12,14,15]. Recently, however, studies have shown that some markers such as CD90 or CD105 are not expressed homogeneously in bulk ADSC populations but are expressed in small ADSC subpopulations, suggesting that ADSCs are phenotypically heterogeneous^[5,7,8]. In our current study, we detected ALDH activity as a stem cell marker in murine ADSCs. High ALDH activity has been reported as a marker for cells such as hematopoietic stem cells and cancer stem cells^[10]. However, not many studies have been performed on ALDH activity and cell differentiation potential in MSCs. In one of these studies, Estes *et al.*^[11] showed the presence of a subpopulation

with high ALDH activity in human ADSCs; however, no difference was found in terms of differentiation potential. In our present study, the cultured murine bulk ADSC population contained approximately 15% of the ALDH^{hi} subpopulation. Additionally, in the induction experiment for adipogenic and osteogenic differentiation for each sorted ALDH^{hi} and ALDH^{Lo} subpopulation, significantly higher adipogenic and osteogenic potentials were found in the ALDH^{hi} subpopulation. The ALDH^{hi} subpopulation had higher cell differentiation potential than the ALDH^{Lo} subpopulation. To the best of our knowledge, this is the first report on the functionally distinguishable subpopulation defined by ALDH activity within murine ADSCs.

Relationships between ribosome biogenesis and stem cells have been described only recently. For example, it was reported that the transition from self-renewal to differentiation depends on the enhancement of ribosome biogenesis accompanied by increased protein synthesis in female *Drosophila* germline stem cells^[16]. Slow growth, low biosynthesis and markedly reduced ribosome biogenesis were observed in hematopoietic stem cells that lacked RUNX1, which is known to promote the transcription of essential ribosome-related proteins^[17]. We have few reports about relationship between ribosome biogenesis and MSCs. One of these reports presented one of core proteins of 60S ribosome is necessary for differentiation of osteocyte from MSCs^[18]. In our current study, GSEA revealed the significant enrichment of ribosome-related genes in the ALDH^{hi} subpopulation compared to that in the ALDH^{Lo} subpopulation, suggesting that ribosome biogenesis is part of the mechanism underlying the higher differentiation potential of the ALDH^{hi} subpopulation.

ADSCs can be obtained in a less invasive manner from adipose tissue. Therefore, ADSCs are considered to be a promising source of cell-based therapy in the clinical setting. ADSCs have already been used in clinical studies for cardiovascular disease, breast reconstruction after mastectomy, spinal cord injury, cirrhosis, renal insufficiency, skin fistula after surgery, and skin fistula with Crohn's disease^[4,19,20]. Some of those trials reported the therapy to be safe and effective; however, there is obvious room for improvement. For instance, in a phase 3 trial for therapy with allogeneic expanded ADSCs for treatment-refractory complex perianal fistulas in patients with Crohn's disease (ADMIRE-CD trial), approximately 50% of patients who received ADSC-therapy experienced remissions^[21]. Although this is a significant achievement, further research and development are required in relation to the patients who did not respond to this trial.

Purification of specific subpopulations and engineering of ADSCs into cells that are highly efficient in differentiating into specific tissues might help obtain basic knowledge for cell-based therapy, which is more specific to individual disease conditions of each organ for which ADSCs are used. Further investigation is required to identify the underlying mechanisms that regulate ribosome biogenesis and differentiation in ALDH^{hi} ADSCs.

In conclusion, we demonstrated that murine ADSCs

have a distinct subpopulation defined by ALDH activity. Furthermore, the ALDH^{hi} subpopulation had higher osteogenic and adipogenic differentiation potential than the ALDH^{Lo} subpopulation. Ribosome biosynthesis is suggested to be a remarkable difference between ALDH^{hi} and ALDH^{Lo} subpopulations.

COMMENTS

Background

Adipose-derived stem cells (ADSCs) are recognized as useful materials for regenerative therapy. Recent study revealed the existence of subpopulations in ADSCs by surface antigen markers. However, functions of these markers remain elusive. Aldehyde dehydrogenase (ALDH) activity is commonly used as functional marker to identify human and mouse hematopoietic stem cell, though there has been no report about identification of a subpopulation(s) in murine ADSCs using ALDH.

Research frontiers

Several surface antigen markers are reported to be capable of prospectively identifying distinct ADSCs subpopulations in human and murine. However, the function(s) of those reported markers are poorly understood. ALDH has its known function, such as a protective effect to hematopoietic stem cells through acetaldehyde detoxification, although it is not known in ADSCs.

Innovations and breakthroughs

The authors suggest a novel area of research consisted of ALDH, stem cell, and ribosome biosynthesis, by reporting here ALDH^{hi} murine ADSCs are highly capable of differentiation, and have enriched ribosome-related gene sets.

Applications

The authors current findings of ALDH^{hi} subpopulation of ADSCs might provide future application for enrichment of more useful cells which is applicable to an efficient cell-based therapy. Moreover, by elucidating mechanisms of the higher differentiation potentials shown in ALDH^{hi} subpopulation of ADSCs might provide knowledge of a key regulator(s) of differentiation, and links between ribosome biosynthesis.

Terminology

ADSCs: Adipose-derived stem cells can be obtained from adipose tissues and induced to differentiate into adipocytes, osteocytes and chondrocytes; ALDH: ALDH is a superfamily comprising 20 intracellular enzymes and is responsible for the oxidation of various aldehydes. Some reports identified ALDH is a marker that detect hematopoietic stem cells and cancer stem cells; GSEA: Gene set enrichment analysis is a comprehensive analysis of gene expression by a computational method.

Peer-review

This is a very interesting and well executed piece of work, with suitable controls.

ACKNOWLEDGMENTS

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Amyotrophic lateral sclerosis as a protein level, non-genomic disease: Therapy with S2RM exosome released molecules

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Abstract

Amyotrophic lateral sclerosis (ALS) is a rapidly progressing neurodegenerative disease that leads to death. No effective treatments are currently available. Based on data from epidemiological, etiological, laboratory, and clinical

studies, I offer a new way of thinking about ALS and its treatment. This paper describes a host of extrinsic factors, including the exposome, that disrupt the extracellular matrix and protein function such that a spreading, prion-like disease leads to neurodegeneration in the motor tracts. A treatment regimen is described using the stem cell released molecules from a number of types of adult stem cells to provide tissue dependent molecules that restore homeostasis, including proteostasis, in the ALS patient. Because stem cells themselves as a therapeutic are cumbersome and expensive, and when implanted in a host cause aging of the host tissue and often fail to engraft or remain viable, only the S2RM molecules are used. Rebuilding of the extracellular matrix and repair of the dysfunctional proteins in the ALS patient ensues.

Key words: Amyotrophic lateral sclerosis; Proteins; Stem cells; Extracellular matrix; Exposome; Heat shock protein; Stem cell molecules

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Core tip: The author propose that amyotrophic lateral sclerosis is a disease of damaged and misfolded proteins induced by environmental regulators, occurring at the level of translation and post-translation, and not at the level of the genome. The damaged proteins disrupt the extracellular matrix surrounding neurons, precluding normal communication between neurons and the neural stem cells that surround the neurons. As a consequence, the neural stem cells no longer properly shuttle heat shock proteins to the neurons, and many proteins within the neuron misfold. The misfolded proteins are prion-like, leading to a spreading, destructive sequelae within and between neurons and other cells in the nervous system.

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INTRODUCTION

Epidemiological studies of amyotrophic lateral sclerosis (ALS) are in progress, but so far we know the incidence is related to aging, primarily those over 60, two in 100000 new cases per year, and men are afflicted 1.5 times more than women. Despite suspected clusters being studied in the United States, including around bodies of water where contaminated seafood was ingested^[1] the only cluster of ALS patients was confirmed in the Western Pacific (Guam) in the 1940's where locals were ingesting neurotoxins^[2]. Some physicians report that they are "seeing increasing numbers of younger ALS patients". Agreeing with what the epidemiology suggests, the etiology of ALS is multifactorial, including exposure to various chemicals^[3]. Most ALS experts think these brain afflictions have multiple causes, including genetics, epigenetics^[4,5], poor diet, lack of exercise, stress, and a variety of environmental agents, including pathogens^[6], pesticides, for example, pentachlorobenzene, and other chemicals, such as PCBs and formaldehyde^[7]. A recent New England study found pesticides, solvents, or heavy metals, increased the risk of ALS^[8], and Seneff *et al*^[9] propose that glyphosate (Roundup) causes a number of mechanistic abnormalities associated with ALS, including protein misfolding, and elevated levels of glutamate^[10]. Surprisingly, even silver nanoparticles 100 nm in diameter, commonly used in personal, commercial, and industrial products can, when in aerosol form, be absorbed by the nasal mucosa and travel to the brain where they elicit a microglial, potentially inflammatory, response^[11].

Neurological diseases are very complex, involving many underlying causative pathways, and, as such, using poly pharmacological approaches may increase efficacy when compared with single molecule therapies^[12]. Therefore, neurodegenerative disorders such as ALS might require system therapeutic approaches^[13] such as S2RM technology to obtain long-lasting, efficacious results^[12]. In addition to the motor system dysfunction, deficits in mentation are reported for 50% of the ALS patients^[14] demonstrating the complexity of this disease as measured by clinical endpoints.

The mechanisms underlying the destruction of motor neurons and other cell types in ALS are beginning to be understood, with many recent studies demonstrating key irregularities at the molecular and tissue levels in animal models and human ALS patients. Laboratory evidence suggests that stem cell therapy can positively affect ALS^[15]. I will explain these distinct irregularities, show how the distinct components are interrelated, and then, based on the evidence, provide a rationale for using the stem cell-based S2RM technology to treat

ALS.

In the clinic, the practitioner usually has no way to determine whether the stem cells used for therapy are viable, whether they perfuse to the site of injury following administration, and whether the cells engraft into the tissue and remain functional over time. Limited clinical research suggests that the stem cells often don't engraft and therefore don't remain functional in the patient. Laboratory models with detailed analysis show that stem cell injections into host tissue often fail for various reasons, including programmed cell death (apoptosis), a process that may be activated when stem cell numbers (shown for mesenchymal stem cells) become too high in the tissue^[16]. As a therapeutic strategy, given that 80% or more of the therapeutic properties of stem cells is derived from the release of SRM, the better choice is to use the molecules derived from stem cells grown in optimal conditions in the laboratory. Those molecules can then be more easily, less expensively, and with better efficacy, directly administered to the patient^[17].

Further, recent work in humans shows that stem cell therapy may induce advanced aging because forced cellular regeneration that accompanies re-engraftment induces intrinsic stem cell aging as measured using a *p16* biomarker^[18]. In mouse models, serial transplant readily "exhausts" stem cells evidenced by a diminished capacity to produce new somatic cells^[19]. Using only the molecules released from stem cells means that the S2RM technology will not induced intrinsic aging caused by engraftment of the stem cells. Considering the use of iPSCs for therapeutic use, these cells suffer from genetic and epigenetic reprogramming errors, and the older the patient, the more mutations found in mitochondrial DNA along with increased metabolic problems^[20].

I will now describe the mechanisms underlying ALS, and then propose a treatment regimen based on the use of S2RM technology.

MECHANISMS UNDERLYING ALS

The multifactorial disease ALS is a result of some of the mechanisms listed below. Evidence suggest that these many factors, such as exposure to harmful chemicals during conception, development, and throughout life, coupled with aging, infection, and poor lifestyle can lead to the breakdown of the all-important extracellular matrix (ECM) and peri-neuronal nets (PNN) that support the motor tracts. The breakdown of matrix is likely a generalized phenomenon in ALS patients given that in animal models a breakdown of the matrix in the gut leads to "leaky gut," followed by the induction of inflammation^[21]. At the root of the breakdown of the ECM and PNN is a loss of homeostasis, and particularly a loss of proteostasis, meaning a loss of the building blocks for the ECM and PNN. As a major consequence of the ECM breakdown, heat shock proteins (HSP) and chaperone proteins from neighboring cells cannot

supply the motor neurons with their necessary proteins that enable autophagy, and proper protein folding. These results are deadly for motor neurons.

ALS IS NOT GENETIC, AND MUTATIONS ARE NOT SPECIFIC TO ONE GENE

First, because of overlapping genes, a mutation at one nucleotide can have effects in multiple genes^[22]. The very concept of a gene is in dispute^[23], and the nebulous concept of a gene as a cause for disease is in question^[24], especially given the “buffering” capacity of a redundant system to preclude simple mutations as disease causative agents^[25] so well espoused in a new book by Professor Nobel of Oxford University^[26].

“Genetic Factors Are Not the Major Causes of Chronic Diseases” is the title of highly regarded PLoSOne article by Professor Stephen Rappaport at UC Berkeley^[27]. Indeed because of the failure of genetic studies to predict disease, where over 2000 genome-wide association studies have infrequently found risks greater than 1.2^[24], geneticists are now using whole-genome sequencing (WGS) to search for “missing heritability”. The precision with which we can analyze the genome is very seductive, but as is the case with many seductresses, the outcome may not be positive. Rather than genetics, the empirical evidence suggests that exposures, extrinsic factors, are necessary determinants of disease that may or may not be operating in a background of genetic diversity, intrinsic factors. Thus, the extrinsic factors may be operating “above” the genome, for example, at the level of translation and post-translation. That the symptoms of ALS are largely a result of protein dysfunction is indisputable. Over sixty years ago the Millers^[28] first discovered covalent binding of tissue proteins and carcinogen. Today we understand that exogenous (*i.e.*, xenobiotics) and endogenous chemicals (*i.e.*, oxidative stress factors), such as electrophiles, are a primary cause of protein dysfunction through, for example, covalent modifications^[29]. Clearly genetics alone will not predict disease, whereas the exposome in general, and protein function specifically will be important in understanding many diseases and indications.

Although reports continue to claim genetic causes for ALS, such as mutations in C9orf72^[30], studies of the loss of function of C9orf72 is insufficient to cause motor neuron disease by itself^[31]. Likewise in SOD1 mutant mice, a predominant model for ALS, transcription and splicing are normal, suggesting that the abnormality occurs at the level of translation or post-translation^[32]. Further, proteins, such as SOD1 can become pathogenic through non-heritable modifications such as oxidation, with characteristics similar to the SOD1 mutant in familial ALS, suggesting that conformational abnormalities due to WT-SOD1 protein translation or post-translational events can underlie sporadic ALS pathogenesis^[33]. As MIT’s Evelyn Fox Keller^[23] and Oxford’s Denis Noble^[26] explain so well, mutations in the

DNA often don’t express at the level of protein function, and simple sequence analysis of DNA alone will tell us little or nothing about disease. Let us now look at some of the mechanisms known to underlie ALS.

EXTRACELLULAR MATRIX

First, the extracellular matrix (ECM), particularly the perineuronal nets (PNN), surrounding motor neurons are dysregulated in ALS animal models. Reactive astrocytes and the abnormal accumulation of chondroitin sulfate proteoglycans (CSPGs) in the ECM^[34] may create a microenvironment that is non-permissive for neural regeneration in ALS. For example, proteoglycans, specifically Decorin and Perlecan, control cell autophagy, meaning that these ECM components are important in clearing the motor neuron of misfolded proteins^[35]. Further, breakdown of the ECM will lead to fragments that upregulate kainate receptors and exacerbate excitatory neurotoxicity^[36]. PNNs are specialized for neurons and are condensed versions of ECM, the material that surrounds our individual cells. If these PNNs are restored through mesenchymal stem cell therapy by intrathecal injection (IT), then the motor function of the treated animals can be significantly improved. The mode of action in restoring the PNN is thought to be the release of a multitude of trophic factors, including neurotrophic factors, from the implanted stem cells^[15]. In contrast to the success of this study, and like studies where cells or multiple factors are used, administration of only one neurotrophic factor failed to provide any benefit^[37,38]. Recent safety studies show that administration (IT or IM) of stem cells to ALS patients is safe, and early indications are that some benefit was provided^[39]. The aforementioned data provide excellent evidence that the S2RM methodology will work well in treating ALS.

Evidence suggests that the breakdown of ECM may be generalized phenomenon throughout the body in ALS patients given that the basement membrane breakdowns^[40], as does the matrix in the gut, which leads to “Leaky gut” and an ensuing inflammatory response^[21].

MUSCLE AND NEUROMUSCULAR JUNCTION

Evidence that pathological mechanisms begin at the neuromuscular junction (NMJ) rather than within the motor neurons^[41-43] suggests that ALS may be a distal axonopathy. Cultures of satellite cells from biopsies of ALS patient biopsies have been shown to proliferate similarly to cells obtained from healthy muscle. However, the morphology of the ALS derived cells resembles that of senescent cells^[44]. Myoblasts obtained from ALS patients are unable to normally differentiate into myotubes^[44,45].

Human ALS samples are rare, but autopsy of an ALS patient demonstrated skeletal muscle changes

with clear signs of denervation and reinnervation. However, the patient had normal appearing motor neurons; thus pathological changes in skeletal muscle appear to be present before the motor neurons are affected^[41], thus providing human evidence for the “dying-back” phenomenon. *In vitro* cultures of SCs from ALS patients demonstrate a senescent-like morphology, disturbed differentiation, and an apparent inability to proceed through the myogenic program resulting in a decreased ability to regenerate and mature to functional myofiber^[46]. Proteostasis is critical for maintaining aged satellite cells (muscle stem cell) function, preventing the stem cells from becoming senescent^[47]. Proteostasis is dependent on a normal ECM and cytoskeleton. Exercise has been shown to increase the number of MSCs in muscle, where the MSCs act to release growth factors, inducing muscle regeneration^[48]. At the NMJ, agrin, an ECM protein, is critical to maintenance of the synapse, and can rescue the NMJ in animal models of neuromuscular degeneration^[49]. Agrin is expressed by MSCs, including bone marrow stem cells^[50]. Again, agrin constitutes but one part of maintaining homeostasis at the NMJ, and if used alone to treat ALS, would represent a minimally effective, reductionist treatment regimen.

SUPEROXIDE DISMUTASE

The superoxide dismutase (SOD) protein is misfolded in the motor neurons of ALS patients, which means that SOD will not clear out oxygen radicals developed in the highly active motor neurons - thus, the motor neuron will die. Although some correlations of SOD misfolding with DNA mutations has been reported, Horwich’s lab at Yale has shown that the transcripts in SOD1 mice are largely normal^[32], meaning that the RNA for SOD is normal and that the protein dysfunction is a result of translation, or post-translational mechanisms. Misfolded SOD1 will have many negative consequences, including binding the Voltage Dependent Anion Channel isoform 1 (VDAC1) in mitochondria, creating organelle dysfunction^[51].

TDP43

TDP-43 is a protein that helps to regulate gene expression, including RNA transcription, splicing, transport, and translation. CNS cells, in particular motor neurons, are highly vulnerable to TDP-43 dysfunction. In TDP-43 knockdown mice, the effects are predominantly observed in astrocytes within the spinal cord, suggesting that TDP-43 dysfunction in astrocytes may play an important role in motor neuron degeneration. TDP-43 is misfolded in ALS, and spreads in a prion-like fashion^[52].

TDP-43 can also impair mitochondrial dynamics and function in motor neurons. Previous studies described how ALS-associated mutations transform the cytosolic SOD1 into a membrane-interacting protein, and therefore are then capable of associating with organelles such as mitochondria. Recently the peptides derived

from the TDP-43 prion-like domain were shown to have membrane-damaging capacity^[53].

DJ-1 PROTEIN IS MISFOLDED

DJ-1 responds to oxidative stress to protect cells, *i.e.*, motor neurons, from radical oxygen species^[54,55]. Proper function of this protein is also controlled by Heat shock proteins^[56], that must be donated to the motor neurons from adjacent cells. DJ-1 is also responsible for DNA repair, acting as guanine glycation repair protein^[57]. Thus, if DJ-1 is misfolded, guanine glycation occurs and with resultant mutation frequency, DNA strand breaks, and cytotoxicity.

VGF

Fragment depletion of VGF is likely involved in the onset and progression of ALS. In fibroblasts and plasma samples from ALS patients in an advanced stage, VGF C-terminus peptides were reduced in both fibroblast and plasma. In the G93A-SOD1 mice, the same VGF peptides were decreased in plasma in the late-symptomatic stage, while in the spinal cord down-regulation occurred earlier. Immunohistochemistry studies suggests that a large amount of gray matter is VGF C-terminus positive in control mice (including nerve terminals, axons and some somata of motoneurons), while a significant reduction of VGF peptides has already occurred in the pre-symptomatic stage^[58].

FUS

In a small number of patients, mutations in fused in sarcoma/translocated in liposarcoma (FUS/TLS or FUS) are associated with ALS. Animal models provide evidence that mutant FUS exerts a gain-of-toxic function in the cytoplasm of motor neurons resulting in cellular degeneration. Recombinant FUS proteins perfused into squid axoplasm inhibits anterograde and retrograde transport. Regardless of whether the mutation affects the nuclear localization signal (*e.g.*, R521G or R495X) or the glycine-rich domain (*e.g.*, G230C) of FUS, each of the disease variants tested inhibited FAT. When mutant FUS is mixed with the chaperone Hsp110, the impairment is reduced suggesting that a misfolded FUS is responsible for the gain-of-toxic function^[59].

DIPEPTIDE REPEAT PROTEINS

One of the most common genetic changes associated with ALS and frontotemporal dementia (FTD) are aberrant hexanucleotide repeat expansions in C9orf72. Transcripts containing these expansions undergo repeat-associated non-ATG translation (RAN-T) to form five dipeptide repeat proteins (DPRs). In C9orf72-ALS/FTD patients, DPR aggregates are found throughout the CNS. DPRs can also cause degeneration when expressed *in vitro* in neuronal cultures and *in vivo* in animal models.

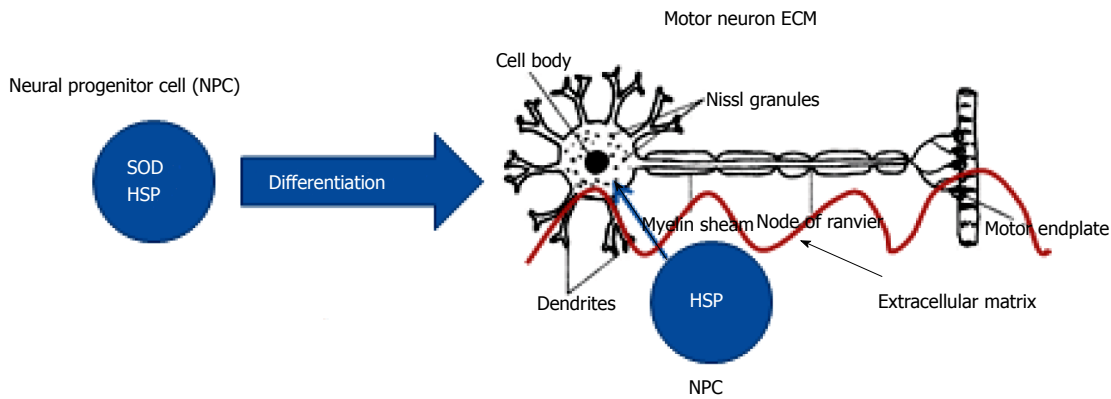


Figure 1 Differentiation of neural progenitor cells into motor neuron. Once differentiated, only SOD produced by motor neuron. HSP must be supplied to motor neuron from surrounding stem cells. ECM facilitates HSP transfer. SOD: Superoxide dismutase; NPC: Neural progenitor cells; ECM: Extracellular matrix; HSP: Heat shock proteins.

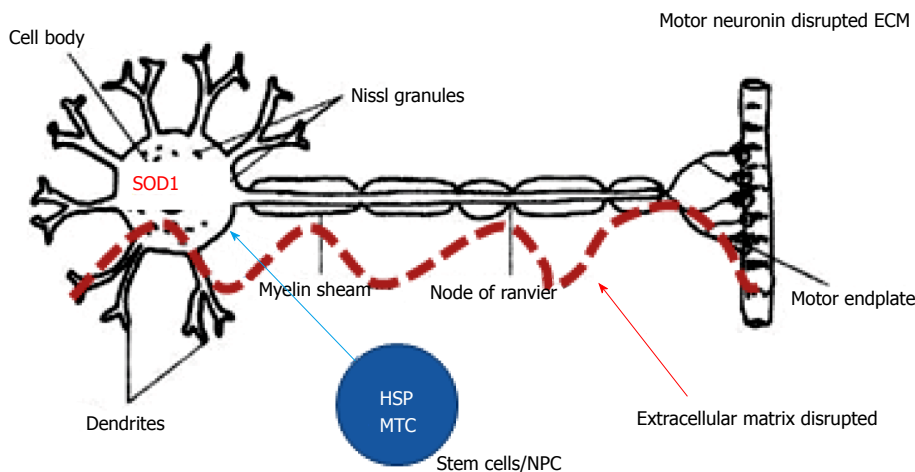


Figure 2 Motor neuron in amyotrophic lateral sclerosis. ALS Motor neurons have misfolded SOD, reduced HSP and mitochondrial function. ALS: Amyotrophic lateral sclerosis; SOD: Superoxide dismutase; SOD1: Superoxide dismutase - misfolded; HSP: Heat shock proteins - disrupted ECM not permissive to HSP transfer; MTC: Mitochondria; ECM: Extracellular matrix.

The prion-like spread of the misfolded proteins is thought to lead to the spreading progression of pathology in the patients of many neurodegenerative diseases. Whether DPRs spread has yet to be determined. Using a number of experimental cell culture techniques, including spinal motor neurons derived from iPSCs of C9orf72-ALS patients, data suggests cell-to-cell spreading of DPRs by exosome-dependent and exosome-independent pathways^[60].

MITOCHONDRIAL DYSFUNCTION

Mitochondria are responsible for efficiently creating energy in our cells, including motor neurons, through a process called oxidative phosphorylation. In ALS, mitochondria function is compromised, and cells go into an inefficient energy producing state called glycolysis^[61] that is 16 times less efficient than oxidative phosphorylation.

Inflammation is an essential component of immunity, but excessive response has been shown to damage tissue and lead to autoimmune disease. The excessive

response is avoided with parallel, integrated regulatory responses that begin the healing. One of the keys to this regulatory response is interleukin-10 (IL-10). IL-10 is particularly important because it limits macrophage proinflammatory functions. IL-10 modifies macrophage function by enhancing the clearance of damaged mitochondria and modulating cellular metabolism to limit inflammation^[62]. IL-10 is part of the molecular mix in the S2RM.

HEAT SHOCK PROTEINS

Neurons, once differentiated from their stem cell precursors, stop producing HSP. Without endogenous HSP, the motor neurons are dependent on surrounding cells to shuttle the HSP from the surrounding cell to the motor neuron (Figure 1). Mesenchymal stem cells release HSP, chaperone proteins^[63], and HSP is transferred by exosomes from one cell to another to maintain protein homeostasis^[64]. A compromised ECM impedes HSP transfer from one cell to another (Figure 2).

EXOSOMES

Secretion of exosomes and their intercellular transmission of chaperone protein are key mechanisms for non-cell-autonomous proteostasis. Hsp40, Hsp70, and Hsp90, are secreted from cells *via* exosomes under physiological conditions^[64]. Thus exosomes from healthy cells, including healthy stem cells, are necessary for the transfer of HSP to motor neurons. Exosome transfer from one cell to another is facilitated by the ECM. Exosomes from MSCs have been shown to alleviate some of the abnormal SOD1 aggregation in neural cells in an *in vitro* mouse model of ALS^[65]. Exosomes that are loaded with the normal set of molecules, and/or the ability of the exosome to travel from healthy stem cells to cells in need of rescue may be compromised in ALS.

GLUTAMATE IS ELEVATED

Astrocytes associated with an ALS mouse model, called AbA cells, do not express detectable levels of the specific glial glutamate transporter GLT-1. Lack of a glutamate transporter in the glial cells of the ALS model may explain, at least partially, excitotoxic damage to motor neurons. Furthermore, the induction of neurotoxicity using the conditioned medium from AbA cells is specific to motor neurons^[66]. As part of the support system, the astrocytes and the ECM, including the PNN, are critical to maintaining glutamate levels surrounding the motor neurons and preventing the glutamate induced excitatory neurotoxicity. Breakdown of the ECM also upregulates kainate receptors that are responsive to glutamate and mediate excitotoxicity^[36].

ASTROCYTES

Astrocytes derived from the spinal cord of persons with sporadic and familial ALS also can kill motoneurons when co-cultured^[67]. AbA cells in culture demonstrate increased proliferation and lack of replicative senescence, thus suggesting aberrant contact inhibition, and therefore a dysregulated ECM that fails to regulate the cells to a normal phenotype^[66].

OLIGODENDROCYTES

Oligodendrocytes are severely affected in ALS and their degeneration has been shown to precede motor neuron death in the mutant SOD1 (mSOD1) mouse model^[68]. Also, oligodendrocyte progenitors rapidly proliferate in the spinal cord of mSOD1G93A mice, but fail to replace degenerating oligodendrocytes, thus leaving motor neuron axons demyelinated. Recent studies suggest that SOD1 in ALS models is directly or indirectly contributing to oligodendrocyte pathology, and suggest that in this cell type some of the damage is irreversible^[69].

PRION-LIKE PROTEINS

Misfolded proteins in the ALS model have similarities to

prions. Key to the SOD1 and TDP43 proteins in ALS is that they self-template themselves, meaning that the proteins replicate themselves without the involvement of DNA and RNA, and then spread to other parts of the nervous system. These prion-like proteins will spread not only throughout the cell, but traveling in exosomes spread from cell to cell^[70]. However, unlike true prions, the ALS prion-like proteins are able to be refolded or cleared from the body, correcting the destructive properties of the protein^[71].

Protein-RNA assemblies serve as the foci of protein misfolding and their maturation into insoluble structures in the ALS state. The assemblies then recruit native proteins, turning them into misfolded forms. This self-perpetuation of misfolding proteins is a "twisted version of classical prion replication" that leads to amplification of pathological protein complexes that spread throughout the neuraxis, offering a pathogenic principle that underlies the rapid disease progression that characterizes ALS, and other neurodegenerative diseases^[72].

CYTOSKELETON

The cytoskeleton in general^[73] and actin^[74], microtubules and neurofilaments^[75] in particular, are believed to be dysregulated in ALS. Understanding that the rescue of neurons through the delivery of HSP and mitochondria is dependent on the ECM and actin-based tunnelling nanotubes (TNT) emanating from the cytoskeleton, and conveyed through the ECM of surrounding healthy stem cells, we can well believe that motor neurons in ALS are put at extreme risk by the interruption of the ECM/cytoskeleton.

BASEMENT MEMBRANE - BLOOD BRAIN BARRIER DYSFUNCTION

Basement membrane dysfunction leading to irregularities in blood/nutrient flow to neurons is a hallmark of macular degeneration, and has recently become evident in ALS^[76]. Here again, breakdown of ECM can lead to vascular irregularities in the motor tracts and induce an inflammatory state. In the healthy adult with a stable ECM and basement membrane on which to attach, microglia are ramified and relatively quiescent. With breakdown of the basement membrane, microglia become mobile and reactive, and can further degrade the ECM^[77]. A chronic para-inflammatory state in the motor tracts ensues. Blood-derived neurotoxic hemoglobin and iron accumulate in the spinal cord, leading to early motor-neuron degeneration in SOD1G93A mice. Chelation of blood-derived iron and antioxidant treatment early in the ALS sequelae mitigated early motor-neuronal injury^[78]. Chronic inflammation with MMP-9 presence, as an example, can lead to laminin-III breakdown and a resulting breakdown of the blood brain barrier. Transplant of bone marrow stem cells into a mouse model of ALS repaired some of the vascular pathology^[76].

ACHE - LOSS OF FUNCTION AT THE SYNAPSE BECAUSE ACHE BECOMES UNBOUND FROM THE ECM AT NMJ

Interestingly, muscle biopsies revealed a reduction in the AChE level of ALS patients^[79], and analysis of the patient's plasma revealed a large increase in circulating AChE^[80]. The increase in circulating AChE may reflect a disruption of extracellularly bound AChE at the NMJ^[81], leading to functional deficits in the muscle. Although in a study where nerve-muscle integrity was altered, and a similar reduction of AChE at the animals NMJ was measured, with an associated increase in plasma levels^[82,83], the exact source of AChE increase in ALS plasma has not been demonstrated. Again, the data are suggestive of an early disruption of ECM, including that of the NMJ.

STRESS

Traditionally we considered inflammation as a defense in response to infection or injury. Inflammation, however, is often induced by tissue stress and malfunction in the absence of infection or overt tissue damage. Stress as a chronic phenomenon, will elicit a chronic, self-perpetuating para-inflammatory state with an active immune response.

Interestingly, activation of autoreactive T-cell receptors by non-commensal microbes might be a common trigger of autoimmune diseases in the immunoprivileged CNS^[84]. This means that stress, and other factors that degrade the microbiota of the gut can have devastating long term effects through chronic inflammation and or elicit a full-blown autoimmune response in the nervous system.

Although the data are poorly collected in third world nations, the incidence of ALS in underdeveloped countries is reportedly lower. This could be due to many factors, including less stress and fewer dangerous chemicals in the environment^[85].

EXPOSOME

Chemical exposure is thought to contribute about 70% of our health state^[86]. Exposure to various chemicals, including PCBs and pesticides has been correlated to human ALS, but not to their matched controls^[3]. The specificity with which pesticides and other environmental chemicals can act to destroy components of the nervous system is exemplified by the chronic exposure of the brain to the lipophilic pesticide rotenone causing a specific loss of dopaminergic neurons^[87].

PGC-1 α

Mesenchymal stem cells better engraft into host tissue when they overexpress PGC-1 α ^[88]. Exosomes from adipose-derived stem cells ameliorate Huntington's disease phenotypes in an *in vitro* model through the

activation of PGC-1 α ^[89]. This has yet to be explored in ALS.

SPATACSIN

In one study of Spatacsin, variants of the spatascin gene were associated with amyotrophic lateral sclerosis neuropathology in one member of one of the 10 families studied^[90]. Given the limited tissue samples available for genetic analysis, and the lack of RNA or protein analysis, while the data warrant further study, no conclusions from this one study can be made.

LEAKY GUT AND DYSBIOSIS

Intestinal homeostasis and the microbiome have recently been shown to play essential roles in neurological diseases, such as Parkinson's disease and ALS. Animal model studies suggests a potential role of the intestinal epithelium and microbiome in the very early progression of ALS^[21]. The ALS mouse model, G93A, which expresses mutant superoxide dismutase (SOD1G93A), showed a number of gut abnormalities. Damaged tight junction structure was present leading to increased permeability. Also, significant reductions in the protein expression levels of tight junction ZO-1 and the adherens junction E-cadherin were seen. Increased numbers of abnormal Paneth cells were reported. Because Paneth cells are specialized epithelial cells that can sense microbes and secrete antimicrobial peptides, they are key to host innate immune responses and helping to shape the gut microbiome. A decreased level of defensin 5 alpha, an antimicrobial peptide, was also found in the ALS intestine. A shift in the profile of the intestinal microbiome was also observed, including reduced levels of *Butyrivibrio Fibrisolvens*, *Escherichia coli*, and *Fermicus*, in the G93A mice. Increased expression of the inflammatory cytokine IL-17, and reduced levels of autophagic lysozyme 1, which leads to a reduced ability to clear misfolded proteins) were additional attributes of a disrupted microbiome. Importantly, the dysbiotic properties of the gut were present when the mice were 2 months old, before any other symptoms of ALS had yet developed. A comparison of healthy subjects vs ALS patients also has shown dysbiosis in the ALS patients with a decrease in healthy microbes and decrease in unhealthy microbes of the intestine^[91].

Dysbiosis of the gut may also be involved in the misfolded alpha-synuclein (AS) and other proteins involved in neurodegenerative disorders that display prion-like transmission of protein aggregation. The role of amyloid proteins made by gut microbiota in the development of ALS was studied using aged rats and transgenic *C. elegans*. Both animals were exposed to *E. coli* producing the extracellular bacterial amyloid protein curli. Rats exposed to curli-producing bacteria displayed increased neuronal AS deposition in both gut and brain. Also observed was enhanced microgliosis

and astrogliosis compared to rats exposed to either mutant bacteria unable to synthesize curli, or to vehicle alone^[92]. The study showed that bacterial amyloid functions as a trigger to initiate AS aggregation through cross-seeding, while also priming responses of the innate immune system.

Relevant to ALS, and neurodegeneration in general, a probiotic mixture of 8 different strains of bacteria, namely *Streptococcus thermophilus* DSM24731, *Bifidobacterium breve* DSM24732, *Bifidobacterium longum* DSM24736, *Bifidobacterium infantis* DSM24737, *Lactobacillus acidophilus* DSM24735, *Lactobacillus plantarum* DSM24730, *Lactobacillus paracasei* DSM 24733, *Lactobacillus delbrueckii* subspecies *Bulgaricus* DSM24734, has shown reparation of leaky gut and a concomitant improvement in a number of neural functions in aged animal models of neurodegenerative disease^[93].

AGE/RAGE

Fibroblasts are abundant in most tissues and in particular the nervous system. They are crucial for homeostatic maintenance and for building the ECM, as well as pathological ECM alterations observed in tissue^[94]. Proteins in the ECM with a long half-life, such as collagen, can nonenzymatically react with high levels of glucose to form advanced glycation end products (AGEs). AGE-modified collagen increases stiffness of the matrix making the matrix resistant to hydrolytic turnover, resulting in ECM proteins accumulating in the microenvironment of cells. In addition, AGE will activate the receptor called RAGE (The receptor for advanced glycation endproducts). RAGE activation in turn can negatively impact normal ECM formation^[95].

Many lines of evidence suggest that inflammatory responses play a critical role in the pathogenesis of motor neuron degeneration in ALS. RAGE is a key component in regulation of both innate and adaptive immunity in different pathologies associated with inflammation. RAGE mediates inflammatory responses and microglia stimulation in the brain^[96], leading to neuronal damage, neurodegeneration, and resulting in symptomatic brain disorders. Immunohistochemical analysis of RAGE in control and ALS patients showed increased expression in reactive glial cells in both gray (ventral horn) and white matter in the spinal cord^[97]. Expression of RAGE is also higher in the SOD1 transgenic mouse model of ALS vs wild-type mouse spinal cord. Further, treatment of SOD1 transgenic mice with soluble RAGE (sRAGE), a natural competitor of RAGE that sequesters RAGE ligands and blocks their interaction with cell surface RAGE, significantly delays the progression of ALS and prolongs life span as compared to vehicle treatment^[98].

Part of the therapeutic strategy of S2RM is to return proteostasis to a normal state using the S2RM technology, thus rebuilding the ECM to a normal state, and reducing the deleterious build-up of AGE and the subsequent activation of RAGE.

WHAT LEADS TO BREAKDOWN OF THE ECM

Environmental toxins, xenobiotic electrophiles for example, can disrupt DNA and protein function and degrade ECM^[99]. Damage to ECM triggers inflammation and further degradation of the extracellular matrix (ECM). The ECM is an intricately arranged biochemical and mechanical scaffold composed of secreted proteins and complex sugars that collectively support cell function and survival. Some ECM molecules become aberrantly expressed following damage, whereas other proteins are cleaved into bioactive fragments known as damage-associated molecular patterns (DAMPs) or "alarmins". The DAMPs are able to bind different types of pattern recognition receptors (PRRs), and can influence the phenotype and magnitude of inflammation. Further, the enzymes and inflammatory mediators released by immune cells resulting from the damage further degrade or alter the composition of the ECM^[100].

Knowing that the breakdown of the ECM/cytoskeleton is a key determinant in ALS, we can then ask, what causes the breakdown of the ECM/cytoskeleton? The exposome, *i.e.*, chemical exposure, is thought to contribute about 70% of health state^[86]. This coupled with lifestyle, stress, and the aging process itself are all risk factors. To summarize, the following factors are known to cause ECM breakdown: (1) Chemical exposure - numerous mechanisms, including breakdown of matrix; (2) stress - signals originating in the sympathetic nervous system can suppress osteoblast function and control the attraction of stem cells to their niche^[101]. Stress, acting through cortisol directly breaks down ECM; (3) aging - degradation of ECM, loss of stem cell function; (4) glycation - binding of sugars with ECM causing ECM dysfunction^[98]; and (5) inflammation, MMPs - chronic para-inflammation or inflammation as a result of ECM breakdown leads to further inflammation and an activation of macrophages (microglia) and a release of many molecules, including MMPs that will exacerbate the inflammatory state thus damaging neurons and muscle.

Collectively these studies suggest that a key determinant of ALS is a dysfunctional ECM and cytoskeleton. I now present the rationale for our therapeutic approach based on the evidence.

S2RM METHODOLOGY FOR TREATING ALS

Simply stated, I believe the predominant cause of ALS is a breakdown of the ECM/microenvironment in the motor neuron tracts of the nervous system; all other mechanism underlying ALS derive from this breakdown. As a major consequence of the ECM breakdown, HSP and chaperone proteins from neighboring cells cannot supply the motor neurons with their necessary proteins that enable autophagy, and proper protein folding. These results are deadly for motor neurons.

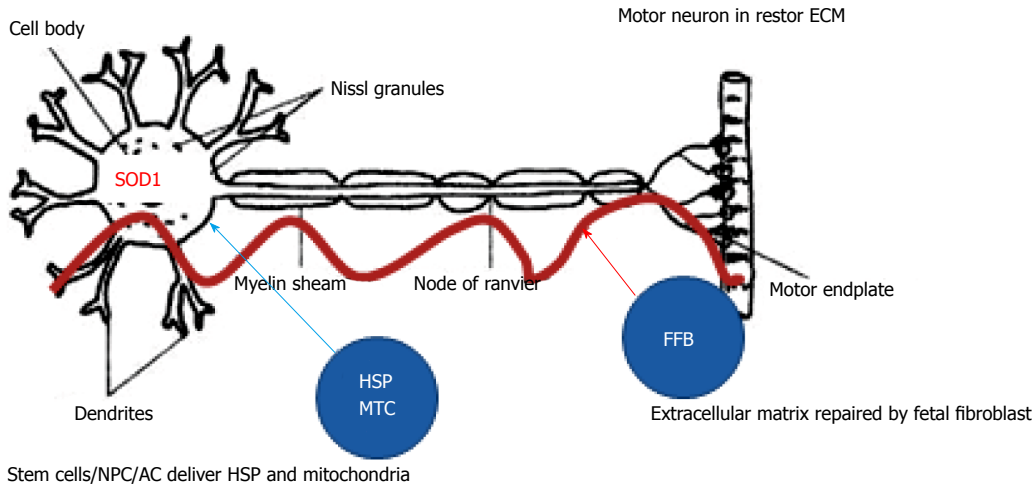


Figure 3 Repairing motor neurons in amyotrophic lateral sclerosis. SOD, HSP and mitochondrial function are restores to motor neurons. SOD: Superoxide dismutase; SOD1: Superoxide dismutase – misfolded; HSP: Heat shock proteins – disrupted ECM not permissive to HSP transfer; MTC: Mitochondria; NPC: Neural progenitor cells; ECM: Extracellular matrix; FFB: Fetal fibroblast; AC: Fetal astrocyte.

My strategy uses a combination of stem cell types to treat ALS. And instead of injecting cells into the patient, we inject the molecules from the multiple cell types into the patient. Here's why. The molecules released from the stem cells are doing all of the work in treating ALS, and the cells are difficult and expensive to work with outside of the laboratory setting, making clinical procedures with the stem cells onerous. Further, once the cells are injected into the patient, the functionality of the stem cells is not measurable, and we know that most stem cells injected into patients are not functional shortly after implantation, failing to make a live graft^[102]. Therefore, our strategy is to grow and stimulate the stem cells in the laboratory to optimize the set of SRM, collect the therapeutic molecules that the stem cells release, and then use those molecules to directly treat the patient. In this manner, we know the "healing molecules" are being delivered to the patient in a defined manner, both in space and in time. The molecules we use are derived from several types of stem cells known to be active in the brain, to stimulate neuron and glial cell growth^[103], build the PNNs, set the ECM to a permissive state for neuro-regeneration, and to correct some of the mechanistic failures and alleviate the symptoms of ALS (Figure 3).

S2RM DELIVERED IN EXOSOMES

As stated above, exosomes are key to this technology given their protective, penetrating, and delivery qualities. They are also immunoprivileged, and have been shown to be used successfully for patient to patient injection without negative immune reaction. Recently, exosomes from human cancer patients have been demonstrated to protect their protein contents from dephosphorylation from endogenous phosphatases. The exosomes were successfully used for protein-based diagnostics of cancer patients for up to 5 years following sampling from the

patient^[104]. This is strong evidence for the ability of our exosome-based therapeutic to be protected in the body as it courses to the target site in the nervous system of the ALS patient.

CELLS USED TO CREATE S2RM-EXOSOME (1-4 ADMINISTERED INTRANASAL, 1-5 ADMINISTERED INTRATHECAL)

First, the use of allogeneic MSCs or their exosomes have been used to treat a number of patients to treat graft-vs-host disease with success, meaning that stem cells themselves, and especially their exosomes, will not elicit a negative immune reaction^[105-107]. Because of their small size, unlike the stem cells themselves, exosomes can easily be sterilized by passing them through a 0.22-µm sterile filter. The cell types used to develop the therapeutic are as follows: (1) bone marrow mesenchymal stem cells (BMSC) - conditioned media induces angiogenesis and rescue mitochondrial function - these cells are stimulated and cultured in a special way, explained in methods section below (Figures 4 and 5); (2) neural progenitor cells - neural growth factors; (3) fetal fibroblasts - less than 24 wk post-gestation (secreting growth factors and building blocks of ECM and PNN, allowing a permissive environment for cellular reprogramming of the afflicted motor neurons and astrocytes); (4) fetal astrocytes - homeostasis of motor neuron support cells; (5) satellite cells (muscle stem cells) - known to express HSP. Administered only at spine through Intrathecal Injection. The SRM from the cells listed as 1-4 are collected and can be dosed intranasally to the patient. The SRM from the cells listed in 1-5 are collected and can be dosed by intrathecal injection to the patient.

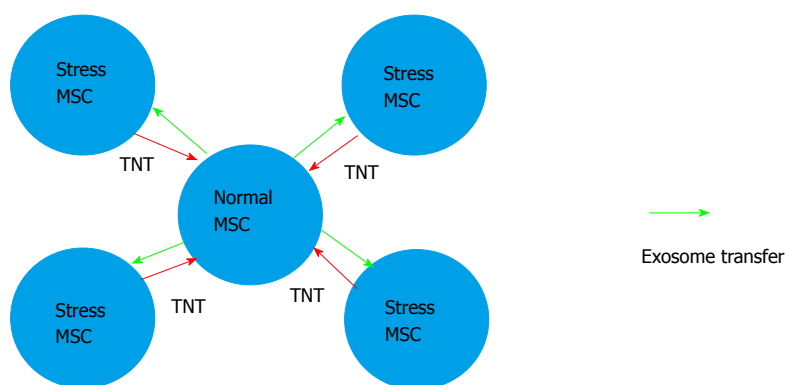


Figure 4 Stimulating and culturing mesenchymal stem cells. TNT formation between stressed and normal MSCs. TNTs facilitate mitochondrial, exosome and molecule transfer between cells. As part of the ECM, the TNTs are extracted with out thermoreversible gel extraction procedure, and included in the S2RM. TNT: Tunnelling nanotubes; ECM: Extracellular matrix.

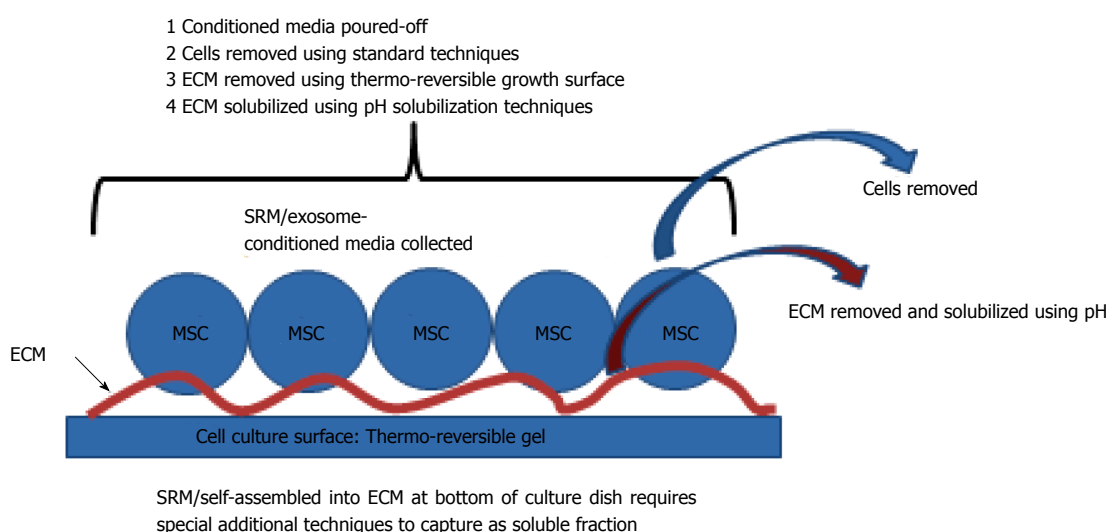


Figure 5 Techniques to capture soluble SRM, exosomes, and self-assembled molecules in extracellular matrix at bottom of culture dish. ECM: Extracellular matrix; MSC: Mesenchymal stem cell.

CAN THE DISEASE BE CURED? CONSIDERATIONS OF ALS AS A CHRONIC PARA-INFLAMMATORY STATE AND HOMEOSTATIC RENORMALIZATION

Tissue damage, independent of infection, such as breakdown of the ECM, Basal Lamina, and PeriNeuronal Nets, can induce a tissue-repair response that involves the activation of both innate and adaptive immunity, such as activation of regulatory T cells, group 2 innate lymphoid cells (ILC2 cells), eosinophils, and M2 macrophages^[108]. This can lead to a state of chronic parainflammation that is self-perpetuating. Let me explain. Between basal homeostatic conditions and true inflammation a “para-inflammation” state exists^[109]. Para-inflammation is an adaptive response of the immune system to low levels of tissue damage, such as in aging whereby oxidative stress or cortisol release for example, accumulates for many decades. The physiological role of para-inflammation is to maintain homeostasis (or re-set the homeostatic threshold of the

tissue) and restore tissue functionality. Unfortunately, because of aging, and living in the industrial world with all of the newly created stressors and an exposome that lacks a corresponding evolved homeostatic renormalization function in our bodies, homeostasis is constantly disrupted. This para-inflammation theory helps to explain many phenomena observed in various chronic disease conditions, an example of which is “inflammaging”^[110]. Why the ECM and Peri-Neuronal Nets will be differentially affected and cause ALS will be hard to figure, but is likely to involve the exposome, what you’ve been exposed to during your lifetime, at conception, during development, and throughout your adult life, along with other lifestyle factors such as stress, exercise, and diet. Further, epigenetic regulation of your parents, and their parents, by what they did and what they encountered in their environments will also have major consequences to your health^[111-114]. This is termed transgenerational epigenetic inheritance. For example, even dietary effects at the epigenetic level can be passed for three generations through an RNA-based mechanism^[115,116], and the drugs consumed

by the father^[117]. The types, quantities, and timing of these factors are likely to be critical in determining whether one will develop ALS, or any other disease of inflammation and aging.

HOMEOSTATIC RENORMALIZATION USING S2RM

The chronic parainflammatory state underlying ALS can best be treated with S2RM technology to renormalize homeostasis. The renormalization process, which includes proteostatic renormalization to rebuild the ECM, TNT, and Peri Neuronal Nets will allow for key HSP and chaperone proteins, along with protein conglomerates called proteasomes, to fold, refold, repair, or expunge the misfolded and damaged proteins, SOD1, DG-1, TDP-43, that lead to ALS. An imbalance in proteostasis leads to many dysfunctions including, for example, inhibition of FGFBP1 expression in muscle cells by increased accumulation of the transforming growth factor- β 1 (TGF- β 1) in skeletal muscles and at their NMJs. In the absence of FGFBP1, NMJs exhibit structural abnormalities^[118].

Further, mitochondrial function will be repaired in the neurons and muscles, and TNTs to transfer healthy mitochondria from healthy cells to compromised cells reestablished - this too is critical in ALS therapy because mitochondria are damaged^[119] by phylogenetically bacterial symbionts of early eukaryotic cells, that when damaged, release mitochondrial damage-associated molecular patterns (formyl peptides and mitochondrial DNA) with evolutionarily conserved similarities to bacterial pathogen-associated molecular patterns. These are released into the circulation and are powerful activators of innate immunity and Nlrp3 inflammasome^[120,121]. Fitting with our hypothesis and treatment strategy, formyl peptides (chemotactic peptides), for example, rearrange the cytoskeleton and change migration patterns of cells, likely interrupting the cell to cell connections that convey HSP, chaperones, exosomes, and mitochondria from healthy stem cells to damaged motor neurons and muscles. These protein factors, along with key microRNA^[122] to renormalize dysregulated microRNA in the ALS patient^[123], contained in the exosomes of S2RM, are the major factors in S2RM therapeutics for ALS.

SPECIAL CULTURE TECHNOLOGY

Our strategy is to use only the molecules that are fully formed and secreted by the stem cells. Crushing the cells and extracting the molecules by harsh techniques is inadvisable as incomplete and misfolded proteins and other molecules may be extracted in this manner. Incomplete and misfolded proteins as we have seen in the aforementioned studies are exactly what we want to avoid in ALS. Stem cells secrete many molecules into the media, and also a rich self-assembly of some of those molecules forms an ECM on the bottom of the culture

dish.

Figure 4 diagrams how HSP is induced in MSCs, and how TNTs are formed between the stressed cells and the normal cells. The culture procedure has two steps: (1) MSCs are cultured under normal conditions, but pulsed with higher temperatures at 39 °C for 24 h in order to slightly stress the cells so the HSPs are formed. On completing of the 24 h stress period at 39 °C, the cells are then removed from the culture vessel using standard techniques so that they can be co-cultured with normal, non-stressed MSCs; and (2) the stressed MSCs are then co-cultured with normal MSCs at a ratio of 1 normal cell to 4 stressed cells. Under these conditions TNTs will form between the normal and stressed cells using actin and microtubules emanating from the stressed cells and guided through the developing ECM at the bottom of the dish that serves as a matrix for the growing cells. In this manner, HSPs, mitochondria, and exosomes can transfer from one cell to another.

Our strategy is to: (1) Capture the conditioned media with all of its soluble molecules, as well as the molecules in the exosomes; and (2) capture all of the molecules that have formed the ECM at the bottom of the dish by the process shown below in Figure 5. Although our therapeutic is protected by issued and pending patents and trade secrets, we can disclose here that our therapeutic is developed using the SRM from five different types of adult stem cells, including lineages from mesenchyme and nervous system.

CONCLUSION

ALS is a disease of protein dysfunction without an RNA or DNA component. Extrinsic factors, including the exposome, cause disruption of proteins that build and maintain the ECM. Once the ECM is disrupted, the support function of stem cells, including neural stem cells, which maintain the proper folding of proteins in the nervous system is degraded. Once the proteins in the nervous system misfold, the misfolded proteins self-template themselves and act in a prion-like manner to spread and destroy neurons. The best way to treat this disease of degraded ECM and misfolded proteins is to restore homeostasis, and in particular, proteostasis, so that the ECM and proper folding of proteins is restored. Proteostasis will allow, for example, tunneling nanotube formation and the transfer of mitochondria from healthy cells to those in need of rescue^[124]. Homeostatic renormalization is accomplished by administering S2RM stem cell molecule technology to the nervous system.

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Pursuing meaningful end-points for stem cell therapy assessment in ischemic cardiac disease

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Abstract

Despite optimal interventional and medical therapy, ischemic heart disease is still an important cause of morbidity and mortality worldwide. Although not included in standard of care rehabilitation, stem cell therapy (SCT) could be a solution for prompting cardiac regeneration. Multiple studies have been published from the beginning of SCT until now, but overall no unanimous conclusion could be drawn in part due to the lack of appropriate end-points. In order to appreciate the impact of SCT, multiple markers from different categories should be considered: Structural, biological, functional, physiological, but also major adverse cardiac events or quality of life. Imaging end-points are among the most used - especially left ventricle ejection fraction (LVEF) measured through different methods. Other imaging parameters are infarct size, myocardial viability and perfusion. The impact of SCT on all of the aforementioned end-points is controversial and debatable. 2D-echocardiography is widely exploited, but new approaches such as tissue Doppler, strain/strain rate or 3D-echocardiography are more accurate, especially since the latter one is comparable with the MRI gold standard estimation of LVEF. Apart from the objective parameters, there are also patient-centered evaluations to reveal the benefits of SCT, such as quality of life and performance status, the most valuable from the patient point of view. Emerging parameters investigating molecular pathways such as non-coding RNAs or inflammation cytokines have a high potential as prognostic factors. Due to the disadvantages of current techniques, new imaging methods with labelled cells tracked along their lifetime seem promising, but until now only pre-clinical trials have been conducted in humans. Overall, SCT is characterized by high heterogeneity not only in preparation, administration and type of cells, but

also in quantification of therapy effects.

Key words: Stem cell therapy; Cardiac imaging techniques; Ischemic cardiac disease; Cardiac regeneration; End-points

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Core tip: Although multiple studies have been published on stem cell therapy (SCT) in ischemic cardiac disease, no universal conclusion regarding its clinical efficacy has been given in part due to the lack of appropriate end-points. A rightful appreciation of SCT impact should be made considering multiple parameters from diverse categories, either objective - evaluating structural and biological functions, or subjective - patient orientated impacting daily quality of life. Current end-points, but also novel parameters investigating molecular pathways and new imaging methods with labelled cells genetically modified are being analytically discussed in this review, disclosing high heterogeneity in SCT efficacy assessment.

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INTRODUCTION

Despite modern cardiac therapies mortality and morbidity caused by ischemic heart disease (IHD) are still high. Rapid blood flow restauration improves the late outcome of patients, but it does not completely hamper myocytes loss or cardiac remodeling. Even if it is not included in the current guidelines for IHD, stem cell therapy (SCT) is one of the latest disclosures in the field.

Since the ambitious beginnings of SCT more than fifteen years ago, numerous heterogeneous results have been published regarding its outcomes. The application of SCT in patients with IHD has been proved clinically feasible and safe by clinical trials aiming heart regeneration. However promising these results may seem, SCT has yet to demonstrate clinical benefit over standard of care^[1,2]. The incomplete profound understanding of cardiovascular regeneration process, inconsistency in study protocols, differences from study to study clinical and biological end-points and the inappropriate routes of delivery, type and dose of cells, patients selection and randomization are some aspects which have delayed its large-scale acceptance by practitioners^[1].

Conducted clinical trials have been comprehensively discussed and analyzed in previous reviews^[3] and meta-analyses (Tables 1 and 2). Analyses were concluded on different number of randomized controlled trials (5-43) including different number of patients (262-2732

patients). Subgroup analyses were centered on various parameters such as left ventricular ejection fraction (LVEF) at enrollment, timing of stem cells (SCs) injection, number of administrated SCs and also patients' age. Since a detailed discussion of utilized methodologies is beyond our topic of interest, we will briefly emphasize their main conclusions: SCT is safe when it is not combined with the administration of growth factors, such as granulocyte colony stimulating factor (G-CSF) that may induce stent restenosis^[4,5], thrombosis^[6] or other adverse events^[7].

Leaving aside the differences in study designs, the lack of a consistent answer to the dilemma concerning the efficacy of cell therapy is sustained by the shortage of adequate end-points and by the shortcomings in evaluating these end-points. Most studies used as evaluation marker LVEF, but it is not sufficient, taking into account that more than 50% of heart failures (HF) caused by IHD have a normal ejection fraction (EF). Other surrogate parameters investigated were: Left ventricular end diastolic volume (LVEDV) and left ventricular end systolic volume (LVESV), infarct size, myocardial perfusion and viability.

So, how can we properly assess the effects of SCT? The first to start with are hard clinical end-points (such as all-cause mortality or cause-specific mortality) employed to conclude whether functional improvement indeed translates into increased survival and reduced morbidity. At that point, other end-points including reinfarction, needed for revascularization and HF worsening can be taken into account. Because the hard primary end-points imply a large number of patients and a long surveillance, composite end-points are an option that overcomes the drawbacks of single end-points. Composite end-points increase the sensitivity of the study, but must be defined in the following manner: Each parameter has to be associated with the primary objective and quantified hierarchically based on its global importance. The foremost disadvantage of composite end-points is heterogeneity in the clinical relevance of the included markers. A solution to counterbalance this inconvenient is to assign a value to each end-point according to its importance, *e.g.*, reinfarction-1, hospitalization for heart failure 0.1, *etc.* This type of hierarchical evaluation proposed by Finkelstein and Schoenfeld^[8] - although controversial - reduces the needed number of included subjects to prove clinical efficiency. For example, a trial can have the following composite end-point with the outcomes ordered by relative severity: Cardiovascular mortality, hospitalization for HF decompensation, 6-min walk test and LVEF or LVESV. Another strategy that may be used for a better assessment is evaluation through multiple parameters from different categories^[9]: Structural measurements (the most frequently utilized group), include LVEF, LVEDV, LVESV, stroke volume, infarct size area, myocardial viability or myocardial perfusion; Biological markers: Brain natriuretic peptide, troponins, cytokines, short and long non-coding RNAs; Physiological determinants: Loading pressures, pressure-volume curves, diastolic function; Functional capacity or

Table 1 Meta-analysis evaluating left ventricle ejection fraction and other outcomes in acute myocardial infarction settings

Ref.	Included studies	Cell type	Pathology	Mean change in LVEF	Other outcomes
Hristov <i>et al</i> ^[104] (2007)	5 RCTs 482 subjects	BMMNCs	AMI	4.21% ($P < 0.00001$)	
Abdel Latif <i>et al</i> ^[105] (2007)	18 trials (RCTs/CSs) 999 subjects	BMMNCs MSCs BM-derived circulating progenitor cells	AMI	3.66% ($P < 0.01$)	Reduced infarct size Reduced LVESV
Lipinski <i>et al</i> ^[106] (2007)	10 trials (RCTs/CSs) 698 subjects	BMMNCs PMCs	AMI	3% ($P < 0.01$)	Reduced infarct size Reduced LVESV Reduced recurrent AMI
Martin Rendon <i>et al</i> ^[107,108] (2008)	13 RCTs 811 subjects	BMMNCs	AMI	2.99% ($P = 0.0007$)	Reduced LVESV Reduced LVEDV
Zhang <i>et al</i> ^[109] (2009)	7 RCTs 660 subjects	BMMNCs	AMI	4.63% ($P = 0.01$)	Reduced MACE
Bai <i>et al</i> ^[110] (2010)	10 RCTs 814 subjects	BMMNCs	AMI	3.79% ($P < 0.01$)	
Takagi <i>et al</i> ^[111] (2011)	15 RCTs 877 subjects	BMMNCs	AMI	2.87% ($P < 0.00001$)	Reduced LVEDV Reduced LVESV
Kuswardhani <i>et al</i> ^[110] (2011)	10 RCTs 906 subjects	BMMNCs Nucleated BMCs BMCs MSCs	AMI	2.07% ($P = 0.008$)	Reduced LVEDV No reduced mortality Reduced recurrent MI and rehospitalization for HF
Clifford <i>et al</i> ^[70] (2012)	33 RCTs 1765 subjects	BMMNCs BM-CD34+ BM-CD34+CXCR4+ MSCs BM-CD133+ BM-CD34+	AMI	2.87% maintained at 12-61 mo	Reduced LVESV Reduced LVEDV Reduced infarct size
Zimmet <i>et al</i> ^[111] (2012)	29 RCTs 1830 subjects	BMMNCs	AMI	2.70% ($P < 0.001$)	No reduced LVEDV No reduced LVESV
Chen <i>et al</i> ^[112] (2013)	5 RCTs 510 subjects	BMMNCs	AMI	4.18% ($P = 0.0002$)	No reduced LVEDV
Jeong <i>et al</i> ^[113] (2013)	17 RCTs 1072 patients	BMMNCs	AMI	2.51% ($P = 0.0002$)	Reduced LVESV Reduced LVEDV
Delewi <i>et al</i> ^[114] (2013)	24 RCTs 1624 subjects	BMMNCs BM-CD133+ BM-CD134+ BM-CD34+/CXCR4	AMI	2.23% ($P < 0.01$)	Reduced LVESV at 6 and 12 mo Reduced recurrent AMI Reduced readmission for HF, unstable angina/chest pain No reduction in infarct size No reduction in LVEDV
Jong <i>et al</i> ^[18] (2014)	30 RCTs 2037 subjects	BMMNCs MSCs BM progenitor cells	AMI	2.10% ($P = 0.004$)	Reduced LVESV Reduced infarct size No reduced LVEDV/LVESV (MRI) No reduced infarct size (MRI) No effect on MACE at 6 mo
Liu <i>et al</i> ^[115] (2014)	8 RCTs 262 subjects	MSCs BM-CD34+ BM-CD133+ BM-CD133+ CD34+	AMI	3.17% ($P = 0.02$)	A trend toward reduced LVESV Reduced MACEs
Delewi <i>et al</i> ^[116] (2014)	16 RCTs 1641 subjects	BMMNCs CD34+/CXCR4+ Nucleated BMCs	AMI	2.55% ($P < 0.001$)	Reduced LVEDV Reduced LVESV
Gyöngyösi <i>et al</i> ^[117] (2015)	12 RCTs 1252	BMMNCs BM-CD34+CXCR4	AMI	No improvement	No impact on MACE No reduction on LVESV/LVEDV
Fisher <i>et al</i> ^[17] (2015)	41 RCTs 2732 subjects	BMMNCs BM-CD34+ BM-CD133+ MSCs	AMI	No improvement in LVEF measured by MRI; 2%-5% increase by echo, PET CT and LV angiography	No reduced MACE No effect on morbidity, quality of life/performance
Cong <i>et al</i> ^[112] (2015)	17 RCTs 1393 subjects	BMMNCs BM-CD34+	AMI	2.74% ($P < 0.00001$, 3-6 mo) 5.1% ($P < 0.00001$, 12 mo)	Reduced LVESV at 3-6 mo Reduced WMSI at 3-6 mo

Lee <i>et al</i> ^[118] (2016)	43 RCTs 2635 subjects	BMMNCs BM-CD133+ BM-CD34+	AMI	2.75% ($P < 0.001$) 6 mo 1.34 % ($P = 0.03$) at 1 yr	No reduced infarct size at 6 mo Reduced infarct size at 1 yr No reduced infarct size at 3 or 5 yr
		MSCs		No reduction at 3 and 5 yr	No reduced mortality at 6 mo and 1 yr Reduced all-cause mortality at 5 yr

AMI: Acute myocardial infarction; BM: Bone marrow; BMCs: Bone marrow cells; BMMNCs: Bone marrow mononuclear cells; CSs: Cohort studies; CXCR4: Chemokine receptor type 4; BM-EPC: Bone marrow endothelial progenitor cells; LVEDV: Left ventricular end-diastolic volume; LVEF: Left ventricular ejection fraction; LVESV: Left ventricular end-systolic volume; MACE: Major adverse cardiac events; MSCs: Mesenchymal stem cells; PMCs: Peripheral mononuclear cells; RCTs: Randomized control trials; WMSI: Wall motion score index.

Table 2 Meta-analysis evaluating left ventricular ejection fraction and other outcomes in chronic, or chronic and acute settings

Ref.	Included studies	Cell type	Pathology	Mean change in LVEF	Other outcomes
Wen <i>et al</i> ^[119] (2011)	8 RCTs 307 subjects	BMMNCs BM-CD34+	CIHD HF	8.40% ($P < 0.01$)	Reduced LVESV Reduced LVEDV
Zhao <i>et al</i> ^[120] (2011)	10 RCTs 422 subjects	BM-CD34+/ CD133+ BMMNCs CPCs	CIHD	4.02%	Reduced LVEDV Reduced LVESV
Donndorf <i>et al</i> ^[121] (2011)	6 trials (4 RCTs and 2 CSs) 179 subjects	BMMNCs BM-CD34+ BM-CD133+	CIHD	5.40% ($P = 0.09$)	No reduced LVESV No reduced MACEs
Jeevanantham <i>et al</i> ^[122] (2012)	50 trials (RCTs, CSs) 2625 subjects	BMMNCs BM-CD133+ and/or BM-CD34+ MSCs MSCs and EPCs	AMI CIHD	3.96% ($P < 0.00001$)	Reduced infarct size Reduced LVESV Reduced LVEDV
Jiang <i>et al</i> ^[123] (2010)	18 RCTs 980 subjects	BMCs BMMNCs MSCs	AMI or CIHD	2.93% ($P < 0.00001$)	Reduced LVESV Reduced LVEDV Reduced infarct area
Cheng <i>et al</i> ^[124] (2013)	5 RCTs 210 subjects	BMMNCs SM	Chronic ischemic HF	No significant increase	Increased 6-min walk distance Improved MLHF score Reduced NYHA class No reduce in all-cause mortality Reduced LVESV
Kandala <i>et al</i> ^[125] (2013)	10 RCTs	Unselected BMCs Enriched BMCs	CIHD	4.48% ($P < 0.0001$)	Reduced LVEDV Improved perfusion
Sadat <i>et al</i> ^[126] (2014)	32 trials (24 RCTs and 8 non-RCTs) 2306 subjects	BMMNCs BM-CD34+ BM-CD133+ CPCs HSCs MSCs	ACS and CAD/HF	4.6 ± 0.7 ($P < 0.05$)	
Xu <i>et al</i> ^[127] (2014)	19 RCTs 886 subjects	BMMNCs CD133+ CD34+ Circulating CPCs Peripheral blood SCs	CIHD	3.54% ($P < 0.001$)	Reduced LVESV No reduced LVEDV
Tian <i>et al</i> ^[128] (2014)	11 RCTs 492 subjects	BMMNCs CD34+ ALDH CD133+	CIHD	4.91% ($P < 0.00001$)	Reduced LVESV Reduced LVEDV
Fisher <i>et al</i> ^[129] (2014)	23 RCTs 1255 subjects	BMMNCs CPCs HSCs MSCs	CIHD HF	2.62% ($P = 0.02, \geq 12$ mo)	Reduced mortality Reduced hospitalization HF (≥ 12 mo) No effect on mortality, rehospitalization for HF at short term (< 12 mo)

					Reduced LVESV Reduced stroke volume index (≥ 12 mo) Reduced NYHA class Reduced CCS score Reduced mortality Reduced rehospitalization for HF Improved performance status Improved QOL Reduced BNP
Fisher <i>et al</i> ^[67] (2015)	31 RCTs 1521 subjects	BMMNCs BMMNCs/CPCs BM-CD34+ MSCs BMMNCs (enriched CD34+) CSCs BM-EPCs BM-CD133+ SM ALHDs ADRCs	HF	2.06% ($P < 0.0001$)	
Rendon <i>et al</i> ^[130] (2016)	6 systematic reviews	BMMNCs BM-CD133+ and/or BM-CD34+ MSCs BM-EPCs Peripheral blood- derived cells CPCs SM ALHDs ADRCs BMMNCs (enriched CD34+)	IHD AMI HF	No significant increase in LVEF in IHD/HF	Reduced mortality in IHD/HF No reduce mortality in AMI
Fisher <i>et al</i> ^[80] (2016)	38 RCTs 1907 subjects	BMMNCs MSCs BM-CD133+ BM-CD34+ CPC ALDH	CIHD HF Refractory angina	Improvement (MRI analysis) on short-term No improvement on long-term	Reduced mortality (≥ 12 mo) Reduced non-fatal AMI Reduced arrhythmias risk No reduced rehospitalization for HF No reduced MACE Reduced long-term
Fisher <i>et al</i> ^[81] (2017)	38 RCTs 1907 subjects	BMMNCs Progenitor cells	CIHD HF Refractory angina	Improvement (MRI analysis) on short-term No improvement on long-term	mortality Reduced refractory angina Reduced non-fatal MI Reduced arrhythmias Reduced rehospitalization for HF/MACE No impact on QOL Improved exercise capacity at long-term

ACS: Acute coronary syndrome; ADRCs: Adult adipose-derived regenerative cells; ALHDs: Aldehyde dehydrogenase positive stem cells; AMI: Acute myocardial infarction; BM: Bone marrow; BMCs: Bone-marrow derived cells; BM-EPCs: Bone marrow endothelial progenitor cells; BMMNCs: Bone marrow mononuclear cells; CAD: Coronary artery disease; CCS: Canadian Cardiovascular Society grading of angina pectoris; CIHD: Chronic ischemic heart disease; CPCs: Cardiac progenitor cells; CSs: Cohort study; CSCs: Cardiac stem cells; HSCs: Hematopoietic stem cells; HF: Heart failure; LVESV: Left ventricular end-diastolic volume; LVESV: Left ventricular end-systolic volume; LVEF: Left ventricular ejection fraction; MACE: Major adverse cardiac events; MLHF: Minnesota living with heart failure questionnaire; MSCs: Mesenchymal stem cells; QOL: Quality of life; RCTs: Randomized control trials; SM: Skeletal myoblasts.

performance status: 6-min walk test, maximal oxygen consumption ($VO_{2\max}$) - the evaluation category with the most important impact from the patient point of view.

Quality of life

Major Adverse Cardiac Event (MACE) composite end-point with no strictly delimited parameters.

STRUCTURAL END-POINTS

While being the most currently used, imaging techniques

are extremely useful for assessing mainly the structural effects of SCT. The majority of studies concentrated on the following outcomes: LVEF, infarct size, myocardial perfusion and viability.

LVEF and left ventricular volumes

The generally measured end-point for assessing SCT outcomes is LVEF. The first clinical studies utilized unselected mononuclear bone marrow or peripheral SCs injected intracoronary. Meta-analyses dealing with these type of cells in acute myocardial infarction (AMI)

settings showed a modest increase in LVEF evaluated by various methods, between 2%^[10,11] and 5%^[12]. One of the arguments against SCT was that the observed differences, albeit being statistically significant, had no clinical benefits. Although the LVEF recovery was small in the early period and not every time sustained, it may induce long-term positive outcomes. In this regard, it is mandatory to assess the effect of SCT on long-term, but few trials extended the follow-up after the period of one year^[13-15]. An additional key aspect depicted by REPAIR-AMI was the importance of timing from AMI until cell injection. It seems that later infusion of SCs has a better outcome (*i.e.*, LVEF) compared with the treatment administered within 4 d. This may be explained by the hostile environment which hampers cell viability due to the presence of inflammatory cells recruited in the injured area; on the other hand, a prolonged interval after AMI is inappropriate for cell transplant as a scar tissue forms and the lack of a proper vascularization also impairs SCs survival. Different imaging techniques were used to determine LVEF: Left ventricular (LV) angiography, radionuclide ventriculography, echocardiography, gated Single-Photon Emission Computed Tomography (gated-SPECT) or magnetic resonance imaging (MRI). The most accurate method to quantify LV volumes and EF is MRI and more recently, 3D-echography^[16]. In Fisher's meta-analysis it can be seen that LVEF improved in the studies that employed echography, gated SPECT or ventriculography, but not in the trials that used MRI imaging^[17]. LVEF increase is a time-dependent process; some meta-analyses investigating SCT in AMI exposed an enhancement in LVEF on short-term, but not on the long-term, explained at least in part by the increase in LV volumes over time^[18].

One aspect being imputed to cell based therapy and an important drawback is the targeted population, the included subjects being not very sick, with baseline LVEF around 50%. The largest trial in AMI settings (BAMI, NCT01569178) is planning to shed light and answer the question if SCT reduces all-cause mortality in patients with impaired systolic function (LVEF < 45%) when compared to a control group of patients undergoing best medical care. According to the Task Force of the European Society of Cardiology, it is the only clinical study able to answer the question if autologous unfractionated bone-marrow offers supplemental advantages on top of AMI standard of care^[19]. Unfortunately, there are no such studies on HF or chronic myocardial ischemia.

Myocardial deformation

The standardization of other modern techniques such as strain/strain rate or tissue Doppler echocardiography are mandatory requests to find a more sensitive and specific marker for SCT outcomes. There are already small clinical trials indicating that tissue^[20] and strain Doppler^[21] assessment of regional systolic function might be more sensitive than global LVEF for the evaluation of SCT after AMI. The concept of myocardial

strain was extended from echocardiography also to MRI detecting subtle improvements in myocardial function earlier than commonly used methods for myocardial function assessment. Myocardial MRI strain imaging has been evaluated only in one study, but when assessed showed significant increment in circumferential strain in the myocardial segments adjacent to the infarction area^[22].

Infarct size

There are several techniques that allow the quantification of the infarction area, either directly, such as nuclear imaging with Positron Emission Tomography-PET or SPECT, contrast-enhanced MRI, or indirectly, appreciating the extent of LV impairment (cine MRI, 2D-3D echocardiography, LV angiography). From the aforementioned approaches the most accurate is contrast-enhanced MRI and the only one capable to distinguish the transmural from the subendocardial infarction. Although the majority of studies using MRI assessment proved no decrease of infarct size compared with placebo^[23,24], there was one trial that interestingly showed a greater reduction in the infarction area in patients having a higher percentage of CD45⁺CD31⁺ cells in the bone marrow. These findings endorse the conclusion that cells' phenotype, as well as their functional capacity are key determinants of individual responses to SCT^[25].

Studies using SPECT disclosed a significantly reduced number of myocardial scar segments per patient in case of intracoronary infusion of an autologous population of culture expanded mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs)^[26], while transplantation of unselected bone marrow mononuclear cells had no impact on the above cited parameter^[27].

Myocardial viability

The current imaging techniques for appraising myocardial viability are: Nuclear imaging (PET or SPECT), low-dose dobutamine echocardiography and MRI. From the practical point of view, an ideal device is the one that provides real-time information as regards myocardial viability and allows targeted cell delivery. Cardiac electromechanical mapping solved this problem and significantly correlates with PET, because in the same time it can be seen if electrical activation translates into mechanical contraction; studies relying on this method showed that SCT increases the local shortening in the infarcted area^[28,29]. Nuclear perfusion imaging, mainly PET-CT has been considered a gold standard for the detection of viable myocardium. Other techniques such as SPECT reported no change compared with control groups^[30-32]. On the other hand, studies using ¹⁸F-FDG PET indicated a gain in myocardial viability^[29,33-36] which was not every time confirmed in trials with low-dose dobutamine echocardiography, possibly due to the fact that severe damaged myocardium can still preserve glucose uptake whilst the contractility is lost^[37]. Low-dose dobutamine echocardiography showed no improvement in the contractile reserve of patients with AMI and mononuclear bone marrow SCT compared

with the non-treated^[34,38], however when MSCs and EPCs were used an increased number of viable segments was observed^[26].

Myocardial perfusion

The available tools for myocardial perfusion evaluation are: MRI (rest first-pass perfusion and late gadolinium enhancement imaging), nuclear imaging (SPECT, PET) and contrast echocardiography. The majority of studies that used SPECT showed a slight increase in myocardial perfusion^[39,40], although more specific and sensitive methods such as PET^[41,42] failed to prove significant difference between SCT and control groups. MRI studies also revealed no improve in perfusion after SCT^[43,44]. PET has the additional benefit of quantifying myocardial blood flow (MBF) using ¹³N-ammonia, ¹⁵O water or ⁸²Rb. MBF quantification is a useful tool to identify patients with balanced triple vessel disease and to diagnose endothelial dysfunction^[45]. One study successfully assessed cardiac perfusion, metabolism, and function in patients treated with intracoronary injection of endothelial progenitors using ¹³N-ammonia and ¹⁸F-FDG PET^[42], showing that selected bone marrow-derived CD133⁺ cells significantly reduced the number of scarred segments and infarct size along with an increase in MBF. Larger studies are required in order to certify the diagnostic and prognostic value of quantitative MBF in relation to SCT.

BIOLOGICAL END-POINTS

N-terminal pro-brain type natriuretic peptide (NT-proBNP) is an important biomarker in IHD. However, when it comes to compare patients treated with SCT to those without treatment, it does not significantly differ. On a short-term follow-up intracoronary bone marrow mononuclear cells (BMMNCs) therapy does not have any impact on NT-proBNP or inflammatory markers such as IL-6, high-sensitivity CRP (C reactive protein) and TNF- α in ST elevation myocardial infarction (STEMI) patients^[46]. Furthermore, on long-term, it was shown that patients with chronic ischemic HF treated with intramyocardial BMMNCs therapy during coronary artery bypass grafting had not had different levels of NT-proBNP from control patients^[47]. In addition, in a 5-year follow-up study, NT-proBNP used as an objective marker for cardiac function remained significantly low in patients treated with circulating or bone marrow-derived progenitor cells^[24]. Even though it was believed that troponin elevation during SCs harvesting and intramyocardial delivery has no meaningful impact on clinical outcome^[48], latest published data support the hypothesis that high-sensitive troponin T serum levels inversely correlate with cell retention and may regulate the response to SCT in patients with post-infarction HF^[49].

Recent data revealed the implication of several cytokines in the progenitor cell evolution and cardiac function in experimental models, but there is only one study which analyzed it in humans. Shahrivari *et al.*^[50] demonstrated that increased platelet-derived growth

factor BB (PDGF-BB) glycoprotein in the peripheral blood is related to increased bone-marrow function, while high levels of IL-6 is related with bone-marrow impairment. In the same vision, supporting the hypothesis that SCT has a role in maintaining the balance of inflammatory markers, Alestalo *et al.*^[51] investigated the implication of cytokines in STEMI patients undergoing SCT by evaluating the levels of IL-4, IL-10, IL-13, IL-1 β , IL-6, TNF- α and IFN- γ ; obtained results pointed to the conclusion that SCT reduces inflammatory cytokines and promotes anti-inflammatory markers.

PHYSIOLOGICAL END-POINTS

Diastolic dysfunction was associated with neurohormonal activation and also with the severity of coronary disease evaluated by angiography, being in consequence an independent predictor of post-AMI prognosis^[52]. Although the majority of studies with SCT in AMI focused their attention on LVEF and LV remodeling evaluation, few of them investigated diastolic function with heterogeneous findings. In the BOOST study, E/A ratio, deceleration time, diastolic tissue velocities and isovolumic relaxation time were determined; among these, the only parameter positively influenced was E/A ratio, which is not satisfactory taking into account that LV filling patterns have a U-shaped relation with LV diastolic function^[53]. This favorable result was maintained only in the first 18 mo^[54], but not at 5 years^[55].

E/e' has a more linear relation to LV filling pressure and therefore is recommended for the evaluation of LV diastolic function. Four months after cell transplant, Herbots *et al.*^[21] did not identify a statistically significant difference between groups with reference to E/e', but another trial proved an improvement, despite the fact that no comparison with the placebo group was completed^[39]. On the other hand, Beitnes *et al.*^[58] reported a constant decrease in E/A and E/e' ratio along with an increase in deceleration time in both groups, independently of SCT. A meta-analysis that included 6 trials with a total of 365 patients revealed a superior improvement in E/e' ratio at 1 year in the treated group compared with control^[56]. In the study conducted by Yao including patients with chronic myocardial disease it was disclosed that even though there were no significant differences between groups in LV volumes, infarct size or myocardial perfusion, there was an overall effect of SCT on E/A, E'/A' ratio and isovolumic relaxation time at 6 mo follow-up^[57].

There are also a series of negative studies, as ASTAMI, where reduced E/A ratio, increased deceleration time and reduced E/e' were observed in both groups, probably reflecting a decrease in filling pressure^[58].

FUNCTIONAL CAPACITY

Apart from the classic parameters, a small number of trials also included patient-centered end-points evaluating the impact of SCT on status performance and quality of

life.

An indicator of functional improvement after myocardial infarction is the performance status which has been assessed in certain studies by means of the New York Heart Association (NYHA) Functional Classification. The published results did not show improvements in NYHA class between the group receiving cell therapy and the control group^[59-63], but the heterogeneity index of the studies was high ($I^2 = 80\%$) making interpretation questionable^[17].

Other manners to address performance are exercise tests: Treadmill test^[40], 6-min walk test^[64], bicycle ergometer^[59] and symptom-limited maximal exercise test^[65]. A meta-analysis including the previous types of tests exposed no improved exercise tolerance. From the analyzed trials only one displayed higher O₂ consumption and better ventilatory response to exercise^[66]. Meta-analysis conducted by Fisher *et al.*^[67] explored, among other parameters, the effect of SCT on exercise capacity; the authors concluded that patients undergoing SCT had greater performance status, but the measurement scales were different impeding correct interpretation.

However, the relationship between SCT and exercise is bidirectional: It is not only that cell transplant can produce changes in performance status, also exercise influences cells' behavior and clinical outcome. Preclinical studies demonstrated that exercise could increase exogenously infused bone marrow cell retention in mouse myocardium, suggesting that exercise may support SCT^[64]. Hence, we should display more interest in addressing this issue.

QUALITY OF LIFE

Whereas 5 trials have examined the quality of life (QOL) after SCs transplantation in AMI on short term, there is still lack of information on the long-term, just one study reporting end-points at 12 mo^[68]. In this small study with only 26 participants, QOL was significantly improved at one year follow-up. From the 5 trials mentioned above, 3 evaluated QOL with Minnesota Living with Heart Failure Questionnaire (MLHFQ)^[64,68,69] and 2 trials with the Short Form 36 Health Survey^[59,62]. A meta-analysis including only 3 of the 5 trials - due to missing data - did not show a significant improvement on short term in the life quality of treated patients compared with the control^[70]. There were also a few studies in chronic IHD or HF, but due to the fact that the results have not been presented quantitatively but only descriptively, no conclusions can be drawn^[71,72].

Angina frequency is one of the disease-specific health-related QOL (HRQOL) items measured using dedicated instruments^[73], therefore there is no wonder that it has been widely assessed in relation to SCT.

Concerning the frequency of angina, all published trials in unanimity showed a reduction in the number of episodes, reported either by a reduction in the frequency of angina episodes per week^[74] or as the frequency of angina at short-term follow-up^[71,75].

A valuable parameter in evaluating the clinical

assessments of SCT would be the psychological dimension, proved to be an essential factor in cardiac rehabilitation^[76]. A pilot study evaluated the impact of psychological and behavioral factors in patients with AMI undergoing SCT indicated that psychological factors should be taken into consideration in evaluation of the response to SCT^[77].

MACE

One commonly evaluated composite end-point in cardiology research is MACE. Although created to evaluate effectiveness and safety, it is study variable as the outcomes differ from trial to trial and there is no universal definition. Meta-analyses proved that MACE creates high heterogeneity in conclusions between studies according to the parameters taken into account^[78].

There is some evidence indicating that even small improvement in LVEF in AMI patients treated with SCT reduces cardiovascular mortality in the long term. REPAIR-AMI trial at 2 and 5 years follow-up showed beneficial clinical effects in cardiovascular mortality and rehospitalization for HF (4 deaths/100 patients in treated group compared with 14 deaths/100 in the placebo group)^[79]. One important limitation of the mentioned study is related to the small number of events (15 deaths in the placebo group and 7 in BMMNCs group during the 5-year follow-up interval). Of note, enrolled patients had a mean baseline LVEF above 45%, meaning that patients with severe impaired systolic function have not been included, namely the cohort at the highest risk for future adverse cardiovascular events.

Unlike the majority of trials where SCT was applied in AMI settings, most recent meta-analyses conducted in chronic IHD and HF pointed out beneficial clinical effects in long term mortality, without losing sight that the quality of evidence is low^[80,81]. In refractory angina patients candidates for revascularization SCT improved the scores for angina, myocardial perfusion and a composite end-point MACE (myocardial infarction, cardiac-related hospitalization and mortality)^[82].

EMERGING PARAMETRES

In recent studies, it was shown a great interest toward microRNAs (miRNAs) as clinical biomarkers in cardiovascular disease^[83]. MiRNAs are small non-coding RNA molecules implicated in gene expression regulation by suppressing the translation of their target messenger RNAs (mRNAs); they can be released in circulation, easily detected in the plasma and quantified by real-time PCR or microarrays, therefore not hard to obtain and analyzed^[84]. Lately, miRNAs have proved their implication in cardiogenesis and regeneration of cardiac tissue, so it is likely to have a possible impact in patients undergoing SCT.

Schulte *et al.*^[85] outlined the perspective use of miRNAs as biomarkers for diagnosis and prognosis of

HF patients. In a recent published study, Karakas *et al.*^[86] evaluated the prognostic value of circulating miRNAs in a cohort of 1112 patients with acute coronary syndrome or stable angina pectoris and pointed out the potential of miRNAs to predict cardiovascular death in these patients. There has been only one study which performed profiling and validation of circulating miRNAs related to MACE in patients with STEMI, demonstrating that specific miRNAs reflect the clinical outcome after STEMI^[87].

Long non-coding RNAs (lncRNAs) were less studied than miRNAs in cardiac pathology^[88]. Still, it was demonstrated that lncRNAs can predict the prognosis in patients with AMI and HF^[89,90]. One of the advantages of lncRNAs is their ability to differentiate between ischemic and non-ischemic HF compared with miRNA. Also, lncRNAs expression differs with hemodynamic conditions, suggesting that it could be a potential biomarker in evaluating myocardial recovery under mechanical circulatory support^[89,90].

Another parameter to consider could be the impact of SCT on endothelial function. There is robust evidence showing that MSCs restore endothelial progenitor cell function and vasculogenesis, thus improving flow mediated dilatation, decreasing vascular endothelial growth-factor (VEGF) while concomitantly increasing EPC-CFU_{sm} (endothelial progenitor cell colony-forming units smooth muscle)^[91].

IMAGING MODALITIES TO BE TRANSLATED FROM BENCH TO BEDSIDE

Different from the presented imaging techniques that assess only marginally and indirectly the fate of transplanted cells, the ideal imaging modality should be able to provide information about their engraftment, survival, proliferation, differentiation, maturation and integration. Labelling strategies for adequate *in vivo* surveillance and cell tracking is the key to solve some unanswered questions about SCT in cardiovascular diseases and it includes superparamagnetic-iron oxide (SPIO) MRI, direct labelling and reporter genes.

Direct imaging implies cells incubation with various probes that enter the cell by endocytosis (SPIOs), transporter uptake (¹⁸FDG) or passive diffusion (¹¹¹In-ox). Direct labelling of cells using magnetic resonance agents tracks cells and gives details about their biology. SPIO persists in the cells and along with the high resolution and good tissue contrasts make MRI a suitable tool for cell tracking^[92]. A drawback of MRI-SPIO worth considering in long-term imaging is the uptake of the contrast agent in the resident macrophages that can show a false-positive increase of the signal as if there would be high engraftment and survival. This inconvenient of SPIOs accumulation in macrophages is of interest in studies investigating inflammation sites^[93]. What is more, even if there is little or no impact of SPIO as regards cells viability and proliferation capacity, some evidence indicate that

SPIO labeling of MSCs impedes cellular differentiation down a specific pathway (*i.e.*, chondrogenesis but not adipogenesis or osteogenesis)^[94]. Nevertheless, this effect must be product dependent because there are other iron-based products approved that do not illicit harmful effects neither on the hematopoietic, nor on the BM MCSs^[95].

Direct radionuclide labelling is widely spread, has high sensitivity, but poor spatial resolution. SPECT and PET are the most frequently employed to describe bio distribution. When cells are injected into the coronary artery or vein by using the stop-flow technique, the retention of BMMNCs is 10.3% and 3.1%, respectively^[96]. When CD34⁺ cells are labelled with ^{99m}Tc-HMPAO retention rises at 19% at 18 h post-injection^[97]. But an important disadvantage is the short half-lives of the used radiotracers that does not allow long-term follow-up.¹¹¹In-oxine having a T_{1/2} = 2.8 days lengthens the total tracking duration to 3-4 d, pointing a level of 2% cell retain^[98].

Reporter gene imaging needs transfection or transduction with reporter gene constructs. After transcription and translation of the reporter gene under the control of a promoter, reporter proteins cumulate into the cell. Upon insertion of a probe specifically to the reporter gene (optical, radio-labelled), the signal starts to be generated and the cells are detected with different imaging modalities (PET, MRI, SPECT, CT, bioluminescence or fluorescence imaging). Reporter genes for cardiovascular SCT seem to be an ideal approach, but apart from one study^[99] that applied it to cytolytic CD8⁺T_s in a patient with glioblastoma, all other trials were preclinical^[100,101]. Not only distribution and proliferation can be assessed with reporter genes, but also differentiation and maturation of cells using a promoter for a differentiation-specific locus, such as sodium-iodide symporter^[102]. On the other hand, reporter gene technique implies genetic modification that seriously increases the risk for mutagenesis. In order to impede inappropriate insertion and prompt targeted insertion, novel gene editing methods can be used such as transcription activator-like effector nuclease (TALEN) or clustered regularly interspaced short palindromic repeats (CRISPR).

All the aforesaid imaging modalities are valuable tools for *in vivo* surveillance and cell tracking waiting to be refined and translated in clinical practice.

CURRENT RECOMMENDATIONS REGARDING SCT

Recommendations in the field of SCT target preclinical and clinical research and are of great value in the perpetual quest to overcome the above mentioned hurdles. In accordance with the requirements for good clinical practice and clinical research established by the regulatory bodies in the United States and Europe, phase II clinical trials should not only consider a variety of efficacy domains, but also should assess the potential benefits of SCT while not focusing on the statistical significance of *P* value^[1]. Furthermore, they should

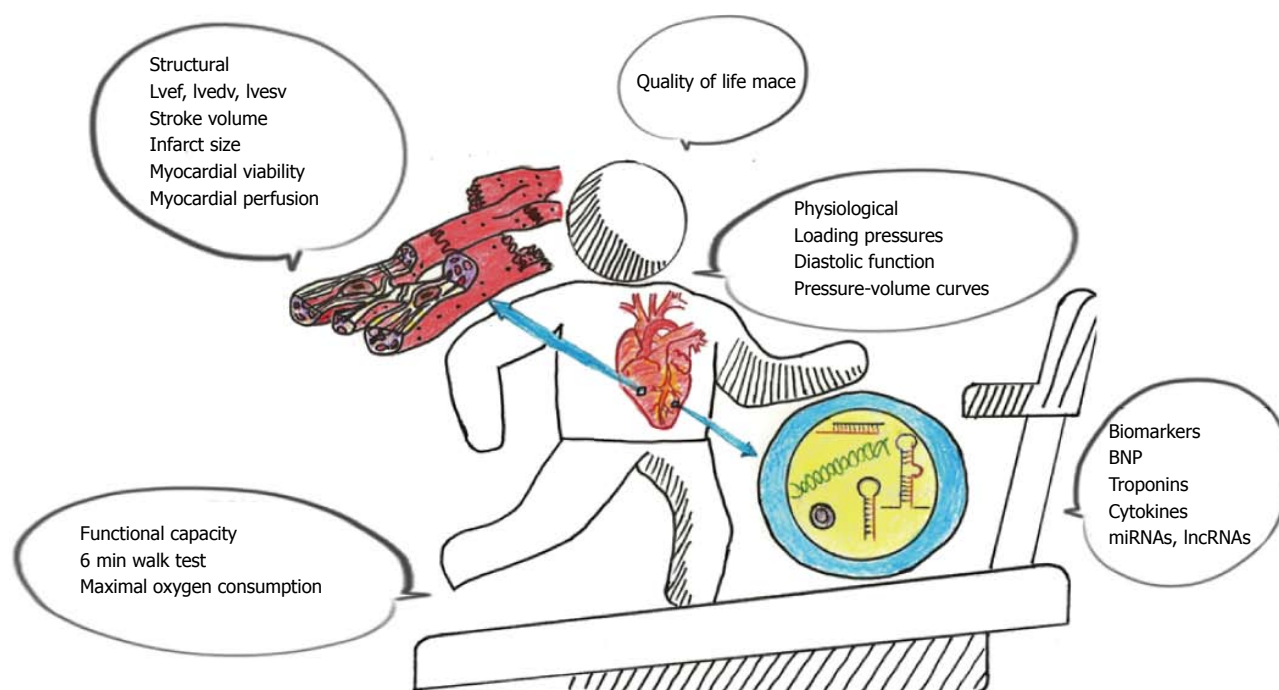


Figure 1 Schematic representation of primary surrogate endpoints grouped by categories. LVEF: Left ventricle ejection fraction; LVEDV: Left ventricle end-diastolic volume; LVESV: Left ventricle end-systolic volume; MACE: Major adverse cardiac events; BNP: Brain natriuretic peptide; miRNAs: MicroRNAs; lncRNAs: Long non-coding RNAs.

include many primary surrogate end-points such as functional and structural measures, biomarkers, quality of life and functional capacity (Figure 1). More precisely, phase II clinical trials have the purpose of generating hypotheses to be used in the appropriate design of pivotal confirmatory phase III clinical trials^[1,2]. Finally, the utilization of hard clinically meaningful end-points is compulsory for the assessment of whether functional improvement positively translates into heightened survival and reduced morbidity^[103]. In this regard, phase III trials should test hard clinical end-points such as all-cause mortality or cause specific mortality, improved survival, reduced clinical events/number of hospitalizations which have applicability in the daily clinical practice. Also, well-designed phase III trials should evaluate subjective clinically relevant end-points as symptom score and HRQOL^[1,2].

Another recommendation is related to the techniques that should be used for surrogate end-points measurements; accordingly, the most reproducible techniques are endorsed (e.g., MRI, PET), while centralized analysis should be settled by core laboratories^[1]. Nonetheless, patient selection is of the essence. When designing a new clinical trial, confounders such as gender, age, comorbidities, concomitant medications, disease vulnerability and severity should always be taken into consideration, if possible by means of predictive scores of outcomes^[1,103]. The focus for inclusion/exclusion criteria in the trial should be on subpopulations with poor prognosis, as they are the target patient that could benefit the most from SCT^[1].

New “mechanistic” end-points are required in order

to better understand the regeneration capacity of the adult mammalian heart and to validate hypothesis on SCs mechanisms of action; these novel end-points should be integrated in traditional safety and efficacy end-points - either surrogate or clinical, only after proper validation in the preclinical research field and in agreement with regulatory recommendations^[1].

With regard to the aforementioned recommendations, in their position paper issued on May 2017, TACTICS highlights the challenges in the field of cardiovascular regenerative medicine for the next decade. Among their global aims are achieving uniformity and, consequently, meeting the required norms for clinical research of animal models for cardiovascular research; using collective achievement of phase III multicenter clinical trials that are optimally designed to improve standard of care in cardiovascular medicine and demonstrate the clinical efficiency of SCT. The last but not the least goal is to certify implementation of accepted SCT *via* transnational standardization of regulatory requirements.

Regarding initiation of future autologous bone marrow cells clinical trials in AMI, the recommendation is to await results from on-going BAMI trial (NCT01569178)-multicenter, randomized, controlled, phase III study-designed to assess efficacy of SCT with concern to morbidity and mortality in patients with reduced LVEF after successful reperfusion when compared to a control group of patients undergoing best medical care.

As for clinical trials in HF, cardiopoietic cells-either primary or engineered - should be used. Taking into consideration the documented safety of SCT approaches, trials evaluating repeated administration should be

studied in order to enhance long term clinical outcome^[19].

CONCLUSION

SCT in ischemic cardiac disease is characterized by high heterogeneity in the assessment of therapeutic benefits due in part to the imprecise end-points. Apart from the classic structural parameters, new emerging imaging or biological markers promise to enlighten the field of cardiac regeneration offering less debatable results.

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Yin and Yang of mesenchymal stem cells and aplastic anemia

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Abstract

Acquired aplastic anemia (AA) is a bone marrow failure syndrome characterized by peripheral cytopenias and bone marrow hypoplasia. It is ultimately fatal without treatment, most commonly from infection or hemorrhage. Current treatments focus on suppressing immune-mediated destruction of bone marrow stem cells or replacing hematopoietic stem cells (HSCs) by transplantation. Our incomplete understanding of the pathogenesis of AA has limited development of targeted treatment options. Mesenchymal stem cells (MSCs) play a vital role in HSC proliferation; they also modulate immune responses and maintain an environment supportive of hematopoiesis. Some of the observed clinical manifestations of AA can be explained by mesenchymal dysfunction. MSC infusions have been shown to be safe and may offer new approaches for the treatment of this disorder. Indeed, infusions of MSCs may help suppress auto-reactive, T-cell mediated HSC destruction and help restore an environment that supports hematopoiesis. Small pilot studies using MSCs as monotherapy or as adjuncts to HSC transplantation have been attempted as treatments for AA. Here we review the current understanding of the pathogenesis of AA and the function of MSCs, and suggest that MSCs should be a target for further research and clinical trials in this disorder.

Key words: Hematopoiesis; Targeted therapies; Stem

cells; Hematopoietic stem cell transplantation; Aplastic anemia; Mesenchymal stem cells

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Core tip: Acquired aplastic anemia (AA) is a bone marrow failure syndrome characterized by peripheral cytopenia and bone marrow hypoplasia and is ultimately fatal without treatment. Our incomplete understanding of the pathogenesis of AA has limited development of targeted treatment options. Here we review the current understanding of the pathogenesis of AA and the function of mesenchymal stem cells (MSCs), and suggest that MSCs should be a target for further trials in AA.

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INTRODUCTION

Aplastic anemia (AA) is an acquired bone marrow failure syndrome characterized by pancytopenia and bone marrow hypoplasia. Patients often become dependent on blood and platelet transfusions and are at risk for significant infections from neutropenia and leukopenia. The natural history of untreated AA is death, most commonly from infection or hemorrhage. Current treatments focus on suppressing immune-mediated destruction of bone marrow stem cells or replacing hematopoietic stem cells (HSCs) by transplantation. However, our incomplete understanding of the pathogenesis of AA has limited development of targeted treatment options. Here we review the current understanding of the pathogenesis of AA and the role that mesenchymal stem cells (MSCs) can play in treatment.

EPIDEMIOLOGY AND DIAGNOSIS

Patients with AA enter medical care after presenting with symptoms related to pancytopenia - fatigue from anemia, bleeding from thrombocytopenia, or infection from neutropenia. The diagnostic criteria for AA include cytopenias and decreased marrow cellularity as noted in Table 1^[1]. Congenital bone marrow failure syndromes such as Fanconi Anemia and Dyskeratosis Congenita can present similarly. However bone marrow failure syndromes such as these can be identified by disease-specific genetic testing that is often performed as part of the initial evaluation of AA. When a genetic mutation is identified that drives the development of bone marrow failure, treatment is directed toward the underlying disease. In acquired AA, no identifiable genetic cause is identified. The scope of this review will focus on the acquired form of AA.

Table 1 Criteria for severe aplastic anemia^[1]

Peripheral blood, CBC findings	
Granulocytes	< 500/cu mm
Platelets	< 20000/cu mm
Reticulocytes	< 1%
Bone marrow biopsy findings	
Hypoplasia	< 25% of normal cellularity
	25%-50% of normal cellularity with
	< 30% hematopoietic cells

Acquired AA can be triggered by exposure to viruses, medications, or noxious chemicals but, for most patients, no inciting event is usually pinpointed. The onset of AA tends to occur in young adults or in elderly patients^[2]. The incidence of AA is higher in Asian populations, affecting 3.9-7 patients per million, compared to European populations where 2-2.4 patients per million are affected^[2]. Males and females are affected equally^[2].

Once a patient is diagnosed with AA, supportive care is initiated and frequent blood and platelet transfusions are performed. Standard-of-care treatment is based on whether the patient has a human leukocyte antigen (HLA)-matched related donor. If a matched donor is available, then definitive treatment with hematopoietic cell transplantation (HCT) is recommended. Patients that undergo matched related donor HCT generally have good outcomes with overall survival approaching 90%^[3]. In patients without an HLA-matched donor, accounting for approximately 70% of patients, unrelated or alternative donor transplants have generally been avoided as first-line therapy given the risk of morbidity and mortality associated with transplantation. These patients are treated with a course of immunosuppression with equine anti-thymocyte globulin (ATG) and cyclosporine A (CSA).

A majority of patients without a matched related donor show an initial response to immunosuppression^[3]. However, approximately 30% of AA patients do not respond to immunosuppression or have recurrence of cytopenias with weaning immunosuppression^[3-5]. For these patients, second-line therapies such as Cyclophosphamide or Eltrombopag or alternative HCT is pursued, using either cord blood, unrelated or haploidentical related donors. An increasing number of AA patients are requiring alternative donor HCT; their outcomes have continued to improve with an overall survival of 80%-90%^[6,7]. In addition, recent studies have shown similar outcomes with upfront matched-unrelated donor HCT and matched-sibling donor HCT, further underscoring the role of HCT in treating AA^[8].

UNDERSTANDING THE PATHOGENESIS OF AA

Although our understanding of the pathogenesis of AA is increasing, it remains incomplete thereby limiting the development and implementation of targeted treatment options for these patients.

Immune-mediated stem cell destruction and impaired hematopoiesis

The observation that many AA patients show clinical improvement in blood counts after treatment with immunosuppression points towards an immune-mediated etiology for the disorder. For example, there is growing evidence that there is increased T-cell activation in patients with AA^[3,4,9-11]. Many scientists are working to identify possible inciting factors triggering aberrant T-cell activation in idiopathic AA patients but the primary cause has not yet been fully elucidated. What is known is that effector memory T-cells, which are known to play a role in autoimmunity, are increased in patients with AA^[9,12]. In addition, CD8+ T-cells are expanded in AA patients and they show restricted T-cell receptor (TCR) expression^[13]. Some studies suggest that the TCRs themselves show increased expression of CD3-zeta and co-stimulatory molecule CD28 promoting T-cell activation^[14], whereas other studies suggest a restricted TCR with decreased CD3-zeta expression but with aberrant activity^[15].

AA patients also show a shift to a predominantly pro-inflammatory Th1 T-cell phenotype^[10,16]; this appears to be at least partly triggered by increased expression of the transcription factor T-bet^[10,11]. These Th1 T-cells, in turn, increase production of interferon- γ (INF- γ)^[10,16]. INF- γ has been shown to impair long-term colony formation by hematopoietic progenitor cells *in vitro* suggesting impaired hematopoietic differentiation potential^[16]. INF- γ also induces HSCs (CD34+ cells) to undergo apoptosis^[9]. In addition to the increased T-cell activation, T-regulatory cells, which have suppressor functions, are decreased in AA patients^[17,18]. By this proposed mechanism, the INF- γ producing Th1 cells deplete the marrow of HSCs, leading to the clinically-apparent pancytopenia and bone marrow hypoplasia that is observed.

Impaired MSC function

MSCs are found in adipose tissue, umbilical cords, and the bone marrow. MSCs have the ability to differentiate into other cell types such as chondrocytes, adipocytes, and osteoblasts, and can self-proliferate, maintaining a phenotype of "stemness"^[19]. Bone marrow-derived MSCs lie within the stroma of the marrow and play crucial roles in immunomodulation and hematopoietic support.

Normal MSC function has been shown to include interactions with various immune cells including T-cells, B-cells, NK cells, and monocytes in *in vitro* studies^[20-24]. In culture, MSCs inhibit proliferation of activated T-cells (both CD4+ and CD8+ cells) by halting cell cycle progression through the G₀/G₁ phase^[20-24]. Although T-cells can be appropriately activated, they enter a state of senescence in the presence of MSCs^[21,22]. This immunomodulatory function relies primarily on secreted factors but is also enhanced by cell-cell contact^[21,22,25,26]. INF- γ has been strongly implicated in this phenomenon as well as indoleamine 2,3-dioxygenase (IDO), hepatocyte growth factor, transforming growth factor β (TGF β), HLA-G5, IL-10, and PGE2^[20-23,27]. In the presence of allostimulated T-cells, MSCs stimulate differentiation of T-cells into

T-regulatory cells, which appears to be mediated by HLA-G^[27]. However, others have challenged this finding^[23]. In addition to inhibition of T-cell proliferation, MSCs similarly inhibit proliferation of resting NK-cells^[23,26] and B-cells^[25]. Monocytes in the presence of MSCs change their phenotype and arrest in G₀; they are unable to differentiate into antigen presenting cells (APCs)^[28,29]. Further on this, MSCs themselves have the potential to act as APCs. At baseline, MSCs have low levels of MHC class I and II expression but this is altered by INF- γ . In the presence of INF- γ MHC class I is upregulated, protecting MSCs from NK-mediated cell lysis^[26]. Although at low levels of INF- γ MHC class II is present, higher concentration of this potent immunomodulatory cytokine lead to downregulation of MHC- II and prevent MSCs from acting as APCs^[30,31].

MSCs are also instrumental in supporting hematopoiesis. Recent *in vitro* 3-D models of the hematopoietic niche have been generated using a bio-derived bone scaffold, MSCs, and osteoblasts, which can independently produce extracellular matrix and secrete cytokines that stimulate proliferation of hematopoietic progenitor cells (HPCs)^[32]. MSCs create a scaffold for HPCs by upregulating adhesion molecules such as integrin subunit beta (ITGB1) and enhance HPC proliferation *via* upregulation of Twist-1 and CXCL12^[33,34].

The above notwithstanding, data on MSC function in patients with AA has been conflicting. Some studies have identified distinctly abnormal MSCs from patients with AA^[35-41]. Gene expression profiling identified over 300 genes that were differentially expressed in MSCs from AA patients compared with healthy controls^[39]. This included upregulation of genes involved in apoptosis, adipogenesis, and the immune response^[39]. Kastrinaki *et al.*^[37] reported increased MSC apoptosis in AA patients. Further, MSCs from AA patients have reduced proliferation potential^[35,38,39], mediated by decreased CXCL12 and FGF1 expression^[36,42]. In addition, a number of studies suggest that MSCs from AA patients show defective differentiation with an increased preponderance to form adipocytes^[37,41]. Patients with AA have decreased GATA2 expression and increased PPAR γ expression in their MSCs, in turn leading to increased adipocyte differentiation^[40]. This has been supported by findings in a mouse model of immune-mediated AA, where inhibition of PPAR γ improves bone marrow cellularity and suppresses T-cell activation and proliferation^[43].

Despite these interesting findings, some other groups have found opposite results - mainly that MSCs maintain their immunomodulatory properties in patients with AA^[44-46]. Indeed, MSCs from AA patients have been shown by some labs to have similar morphology and differentiation potential and inhibit T-cell proliferation similar to control MSCs^[44-46]. The discrepancy between these studies and the ones described above may be related to different patient populations (including limited patient numbers), evaluation at different times during treatment, different culture techniques, and differential analyses performed.

Although the *in vitro* experimental data may be somewhat conflicting, MSCs remain an attractive target for treatment of AA, rooted in their role in the pathophysiology of this disorder. The known function of MSCs and the effect of their dysfunction can connect many observations in AA. For example, when MSC function is impaired, HPCs cannot adequately proliferate, activated T-cells are not suppressed, and the bone marrow architecture changes. We hypothesize that this may correlate with impaired hematopoiesis and pancytopenia, destruction of HSCs by activated T-cells, and increased adipocyte differentiation in a hypoplastic bone marrow - all findings seen in AA patients.

MSCS FOR IMMUNOMODULATORY AND REGENERATIVE THERAPY

MSCs have been utilized in the settings of therapy for other disorders due to their immunomodulatory and proliferative functions. Most attention has been focused on MSCs for the treatment of refractory gastrointestinal graft-vs-host disease (GvHD). MSCs have been shown to be effective in treating both adults and children with steroid-refractory acute gastrointestinal GvHD - with response rates of over 50%^[47,48]. The mechanism for MSC improvement in this disease is thought to be related to immune suppression of allo-reactive T-cells^[48]. There is also the possibility that the MSCs may be aiding in tissue regeneration and healing. Similar to the work in GvHD, MSCs have produced improvements in treatment of refractory inflammatory bowel disease and multiple sclerosis, again likely harnessing their immunosuppressive properties^[49-52]. MSCs have also shown promise in neurologic diseases - repair in spinal cord injuries, stroke and amyotrophic lateral sclerosis - and in cardiac regeneration after infarction or cardiomyopathy^[53-57].

The early phase studies using MSCs have shown a well-tolerated safety profile. No infusional side effects have been noted. There is a theoretical risk of ectopic tissue or tumor formation given the ability of MSCs to differentiate into multiple cell types. However, few case reports have noted this occurring. In addition, when expanding and culturing MSCs, trypsin is used to collect the cells and trypsin has a risk of mutagenesis. Again, there have been no reports of this adverse effect^[58].

MSCs enhance engraftment in HCT for AA

Translation of MSC therapy to AA has been relatively limited. Preliminary studies have attempted to use MSCs as an adjunct to HCT to help enhance engraftment or as primary, monotherapy treatment of AA (Table 2).

The findings that AA patients may have defective MSCs have introduced the possibility of MSC replacement as a therapeutic modality. In the collective pool of patients that go to HCT, AA patients are at high risk of graft failure. There is evidence that supporting patients with HSCs in addition to MSCs will better support

hematopoiesis and engraftment^[59-61]. Initial case reports adding MSCs to transplantation were promising. Luan *et al.*^[61] reported a case of a patient with severe AA that underwent matched sibling cord blood transplant but had delayed engraftment; after giving a cord-blood-derived MSC infusion, the patient began to engraft and pancytopenia improved. Similarly, a report on 2 patients with severe AA who had graft failure after HCT were given second transplant from the same donor with addition of MSCs from a haploidentical maternal donor and they were able to engraft^[59]. MSC infusion has also been used upfront around the time of HSC infusion; this approach shorted engraftment, with neutrophil and platelet engraftment occurring by D12 post-HCT, shorter than historical controls^[60,62,63]. In addition, alternative donor transplants including haploidentical donor HCT had shorter engraftment when MSCs were added to the regimen^[59,62]. A recent phase II study by Liu *et al.*^[64] confirmed these findings when bone marrow-derived MSCs were given with haploidentical HCT, 97.6% of patients had engraftment. These studies have all had small sample sizes but overall reports have not described any significant adverse events and suggest a possible benefit. Similar results were seen with umbilical cord-derived MSC or bone marrow-derived MSCs and with related MSCs and third-party MSCs. Larger randomized trials are needed to further validate these findings^[64-66].

MSCs as monotherapy for AA

It is hypothesized that defective MSCs prevent adequate hematopoiesis and infusion of donor MSCs may create an environment more supportive of hematopoiesis. Most studies of MSC infusions as monotherapy have been performed with patients who have been refractory to immunosuppression. One case report described a 68-year-old patient with refractory AA who was unable to proceed to HCT and received 2 haploidentical, bone marrow-derived MSC infusions from her son^[67]. Unfortunately the patient died from overwhelming infection, but autopsy showed improved bone marrow stroma but without improvement in hematopoiesis^[67]. In another single-arm study, 18 patients were given an infusion of third-party, bone marrow-derived MSCs and 33% of patients showed at least a partial response to treatment, eliminating the need for transfusions^[68]. Another single-arm study used weekly infusions of HLA-matched, bone marrow-derived MSCs but found poor MSC bone marrow engraftment^[69]. However, of the 9 patients, 3 patients did have a partial response and were able to become transfusion independent^[69]. In the largest study to date, 53 patients received bone marrow-derived MSCs from matched, haploidentical, or unrelated donors after *in vitro* expansion^[70]. MSC infusion produced modest responses with an overall response rate in the cohort of 28.4% at 1 year^[70]. These preliminary studies support the concept that MSC replacement can improve bone marrow stroma and may alleviate symptoms in some AA patients. However, larger studies are needed to evaluate the utility of MSCs further.

Table 2 Summary of the clinical uses of mesenchymal stem cells in aplastic anemia

Treatment	Intervention	Goal (s) of therapy	Outcome
MSC as adjunct to HCT	MSCs given in conjunction with hematopoietic stem cell transplantation	To prevent graft failure or shorten time to engraftment	Improved donor engraftment
MSC as monotherapy	MSCs given alone	For primary treatment of AA	Partial response in some patients

MSC: Mesenchymal stem cell; AA: Aplastic anemia; HCT: Hematopoietic cell transplantation.

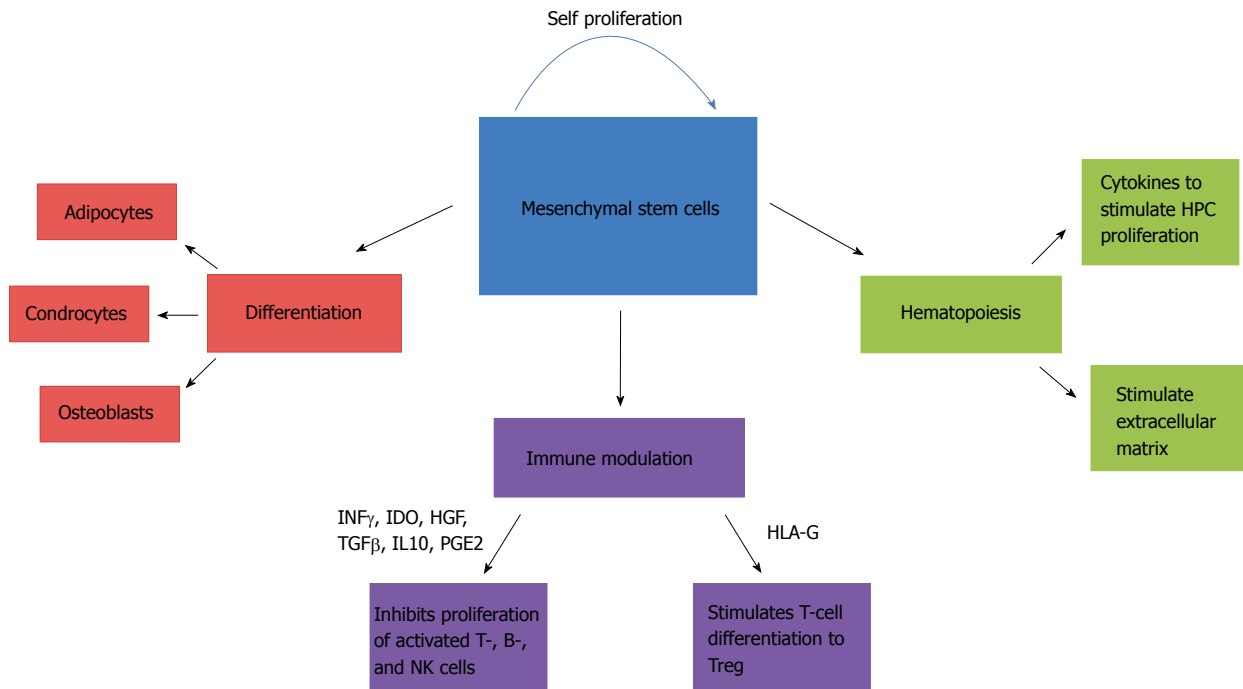


Figure 1 Mesenchymal stem cell function. Mesenchymal stem cells (MSCs) play a role in hematopoiesis, cell differentiation, and immune modulation. MSCs secrete cytokines that stimulate proliferation of hematopoietic progenitor cells and stimulate the production of the supportive extracellular matrix. MSCs inhibit proliferation of T-cells, B-cells, and NK-cells and stimulate differentiation to T-regulatory cells (Tregs). MSCs also have the ability to differentiate into other cell types including chondrocytes, adipocytes, and osteoblasts, and can self-proliferate. For example, when MSC function is impaired, HPCs cannot adequately proliferate, activated T-cells are not suppressed, and the bone marrow architecture changes which may help explain the clinical symptoms seen in patients with aplastic anemia. IDO: Indoleamine 2,3-dioxygenase; TGFβ: Transforming growth factor β; HGF: Hepatocyte growth factor.

DISCUSSION: FUTURE DIRECTIONS FOR RESEARCH AND CLINICAL USE

As we learn more about the biology of AA, the biology of MSCs, the biology of the bone marrow micro-environment, and as we learn how to safely grow and manipulate human cells, we are moving into an exciting phase of personalized biologic therapy for bone marrow failure.

To date, most of the studies referenced in this review point to the promise of MSC therapy in this context. However, these studies have not been sufficiently powered to fully help us understand the role these therapies play in the treatment of AA. As marrow failure is a rare disease, future studies will require novel study design and outcome measures to help the field advance properly. Therefore, basic scientists, cell therapists, and statisticians will be required to join clinicians in developing translational clinical trials that are able to “molecule by

molecule”, “pathway by pathway”, “protein by protein” solve the Rubik’s Cube of an individual’s bone marrow failure and translate that puzzle solving into safe and effective care.

The treatment options are limitless, which is both daunting and exciting. We envision that a patient’s biology will determine what treatments they will be offered. Instead of devising treatments for a heterogeneous disease process, we envision that the genomics and proteomics revolution will lead to an improved understanding of the patient’s individual biology - which will then translate into a rational MSC-based treatment. With such a rare disease as AA, this will require extensive data sharing and evaluation, around the globe, in order to realize the dream of personalized biologic therapy for bone marrow syndromes. Recent breakthroughs in the clinical implementation of gene therapy also offer the possibility of precise modulation of the niche to directly address the unique needs of each patient.

CONCLUSION

Although our understanding of the etiology of AA is increasing, there remains limited development of targeted treatment options. MSCs, which modulate immune response and help enhance proliferation of HSCs, may be an attractive treatment option. Limited studies have shown modest improvement in AA when given as monotherapy and seem to help enhance engraftment when given in combination with HCT. Further clinical research and basic science studies need to be performed in this area.

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Observational Study

High frequency of CD34+CD38-/low immature leukemia cells is correlated with unfavorable prognosis in acute myeloid leukemia

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Abstract

AIM

To evaluate the importance of the CD34+CD38- cell population when compared to the CD34+CD38+/low and CD34+CD38+/high leukemic cell sub-populations and to determine its correlations with leukemia characteristics and known prognostic factors, as well as with response to therapy and survival.

METHODS

Two hundred bone marrow samples were obtained at diagnosis from 200 consecutive patients with newly diagnosed acute myeloid leukemia (AML) were studied between September 2008 and December 2010 at our Institution (Hematology Department, Lyon, France). The CD34/CD38 cell profile was analyzed by multiparameter flowcytometry approach using 8C panels and FACS CANTO and Diva software (BD Bioscience).

RESULTS

We analyzed CD34 and CD38 expression in bone marrow samples of 200 AML patients at diagnosis, and investigated the prognostic value of the most immature CD34+CD38- population. Using a cut-off value of 1% of CD34+CD38- from total "bulk leukemic cells" we found that a high (> 1%) level of CD34+CD38- blasts at diagnosis was correlated with advanced age, adverse cytogenetics as well as with a lower rate of complete response after induction and shorter disease-free survival. In a multivariate analysis considering age, leukocytosis, the % of CD34+ blasts cells and the standardized cytogenetic and molecular risk subgroups, a percentage of CD34+CD38- leukemic cells > 1% was an independent predictor of DFS [HR = 2.8 (1.02-7.73), *P* = 0.04] and OS [HR = 2.65 (1.09-6.43), *P* = 0.03].

CONCLUSION

Taken together, these results show that a CD34/CD38 "backbone" for leukemic cell analysis by multicolour flowcytometry at diagnosis provides useful prognostic information.

Key words: CD34+CD38-/low; Immunophenotyping; Leukemic stem cells; Acute myeloid leukemia; Prognosis

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Core tip: We analyzed the bone marrow samples of 200 acute myeloid leukemia (AML) patients at diagnosis by multicolour flow cytometry and investigated the prognostic value of the most immature CD34+CD38- population. We showed that a higher than > % level of CD34+CD38- blasts at diagnosis was an independent predictor of disease free survival (DFS) and overall survival in a multivariate analysis considering age, leukocytosis,

the % of CD34+ blasts cells, cytogenetic and molecular risk subgroups. Despite heterogeneity and complexity of AML leukemia stem cells, we could still use CD34+CD38- quantification at diagnosis as useful complementary prognostic parameter for risk-stratification AML patients in future clinical trials.

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INTRODUCTION

It has been reported that leukemic cells able to reproduce human acute myeloid leukemia (AML) in NOD/SCID mice are found exclusively in the CD34+CD38- cell compartment^[1,2]. Leukemia initiating cells (LICs) or Leukemia stem cells (LSC) in mice have shown a primitive immunophenotype (CD34+CD38-) with similarities to normal hematopoietic stem cells (HSCs) regardless of the subtype of AML or the immunophenotype of the majority of the leukemic blasts present in the bone marrow^[3-5]. However, other studies showed that LSC were exclusively found in the CD34- compartment. Other investigators described LSC in both the CD34- and CD34+ cell compartments^[6-8].

The importance of the CD34/CD38 subpopulations in the outcome of patients with AML remains controversial. A high frequency of CD34+CD38- immature cell population at the time of diagnosis has been correlated with a higher percentage of chemotherapy-resistant cells and minimal residual disease (MRD)^[9,10].

Previously, it was showed that cancer initiation is fundamentally a dynamic, Darwinian process of mutational diversification and clonal selection^[11]. Most recently, Greaves propose a "back to Darwin" model for leukaemia initiation and development where cells with variable self-renewal potential or "stem cells" are considered as the units of evolutionary diversification and selection^[11]. Independent of frequency of cancer stem cells are in any cancer, if they are the critical cells for therapeutic targeting or control, the dilemma rising and question if their inherent genetic variability makes them a "moving" and therefore "elusive" target considered as the major impediment to successful therapy for advanced or relapsed leukaemia. Therefore, testing of multiple new agents on the backbones of conventional therapies will present serious challenges in the design of futures clinical trials. There is emerging evidence that personalized therapy will ultimately result, adapted for each patient having a unique combination of molecular

features characterizing his leukaemia.

Most recently, Goardon *et al.*^[12] investigated 74 primary human AML patient samples. They showed that the CD34+ cells in about 80% of these cases contained two predominant populations: One CD38-CD90-CD45-RA+ (lymphoid-primed multipotential progenitor LMPP-like cells) and the other CD38+CD110+CD45RA+ (representing granulocyte-monocyte progenitor GMP-like cells). Moreover, these populations showed a hierarchical organization: The CD38-CD45RA+ cells gave rise the GMP-like cells but not vice versa. These results enlarged on the previous view of AML LSC and establish a hierarchy of leukemia populations with decreasing frequency of LSC, implicate normal hematopoietic progenitors, as LMPP and/or GMP as the cell of origin for AML/LSC in the most of cases. One of the most relevant implications of a progenitor phenotype for AML LSC state to the critical stem cell property of self-renewal, the acquisition of self-renewal ability in AML LSC being an aberrant event resulting important genetic and/or epigenetic changes. Taken together, the results of our study emphasize that using CD34/CD38 "backbone" in leukaemia cells analysis by multicolour flow cytometry at AML samples at diagnosis is relatively facile method, rapidly translate in clinical practice as complementary prognostic factor in AML. Multicolour/multidimensional flow cytometry represent very useful tools to identify and characterise immunologic profile of different leukaemia compartments using CD34/CD38/CD45 as "backbone" to design more complex panels (8-10-14 colours) adapted to AML diagnosis and MRD flow evaluation.

The goal of our study was to evaluate the importance of the CD34+CD38- cell population when compared to the CD34+CD38+/low and CD34+CD38+/high leukemic cell sub-populations and to determine its correlations with leukemia characteristics and known prognostic factors, as well as with response to therapy and survival.

MATERIALS AND METHODS

Patients

Two hundred bone marrow samples were obtained at diagnosis from 200 consecutive patients with newly diagnosed AML were studied between September 2008 and December 2010 at our Institution (Hematology Department, Lyon, France). All clinical trials have been considered reviewed and approved by a suitable ethic committee and were completed in accordance with the Helsinki declaration of 1975. All patients signed informed consent according to French legislation.

Multicolor flow cytometry

Briefly, EDTA-anticoagulated fresh bone marrow samples were processed using the whole-blood lysis technique for immunophenotypic analysis. The CD34/CD38 cell profile was analyzed in one single tube containing the following MoAbs: CD7 FITC (clone 8H8.1, Becton Dickinson), CD13 PE (clone L138, Becton Dickinson), CD33 PerCPy5.5

(clone P67.6, Becton Dickinson), and CD34 APC (clone 8G12, Becton Dickinson), CD38 PEcy7 (clone HB7, Becton Dickinson), CD45 APCH7 (clone 2D1 Becton Dickinson), CD19 Pacific Blue (clone SJ25-C1, Invitrogen). Data analyses were made using FACS Diva software (BD Bioscience). Instrument setup was regularly optimized by analysing Calibrite beads-Rainbows 8 picks beads and CST beads system for checking cytometer stability. The required minimal events of CD34+ was set at 20 and the total number events range between 100000-500000. Isotype IgG staining was used as a negative control for ratio rMFI evaluation. Strategy of gating was based on two distinct analyses: CD45low/SSC total blasts and CD34+/CD45low gated cells from total FSC/SSC viable cells. Within CD34+ compartment we divided three subpopulations: CD34+CD38-, CD34+CD38lo, and CD34+CD38hi, based on intensity of CD38 expression; FMO (Fluorescence Minus One) was used for CD38-level, and hematogones populations for CD38hi level. The stem cell compartment CD34+CD38- contain very few events in some patients but these events should tightly cluster in a FSC/SSC plot and CD45/SSC plot. We also measured the intensity of fluorescence signal for CD38 quantified as rMFICD38 from CD34+ gated cells and rMFICD38 from CD45lo/SSC total blasts cells (Supplemental Figure 1C). We evaluated a comparative analysis between %CD34+CD38- cells and rMFI CD38 intensity in 30 normal bone marrow samples (NBM) from 10 volunteers donors (nBM) and in 20 regenerative bone marrow samples (rBM) from different hematologic diseases obtained after chemotherapeutic treatment and considered in molecular remission status. We observed a strong linear correlation between % CD34+CD38- and rMFI CD38 in all samples, with comparable median in nBM and Rbm (Figure 1B) (*P*-value < 0.0001).

Cytogenetics risk classification and molecular characteristics

Karyotypes were classified into three categories (favorable, intermediate, and unfavorable) according to the Medical Research Council (MRC) classification^[13]. NPM1, CEBPA, FLT3 mutations and ITD, MLL partial tandem duplications and Evi1 and WT1 expression were analyzed as previously described^[14-19].

Statistical analysis

R program (version 2.13) was used for statistical analyses. Non-parametric tests were performed to test the impact of prognostic factors. Correlation tests were computed to compare continuous variables. Survival curves were obtained with the Kaplan-Meier method. Log-Rank test and Cox models were performed on overall survival (OS) and event-free survival for univariate and multivariate analyses, respectively. A cut-off value of 1% was used, corresponding to the median percentage of CD34+CD38 population contained in the total blast cell population gated in the biparametric CD45/SSC plots in all 200 AML patients.

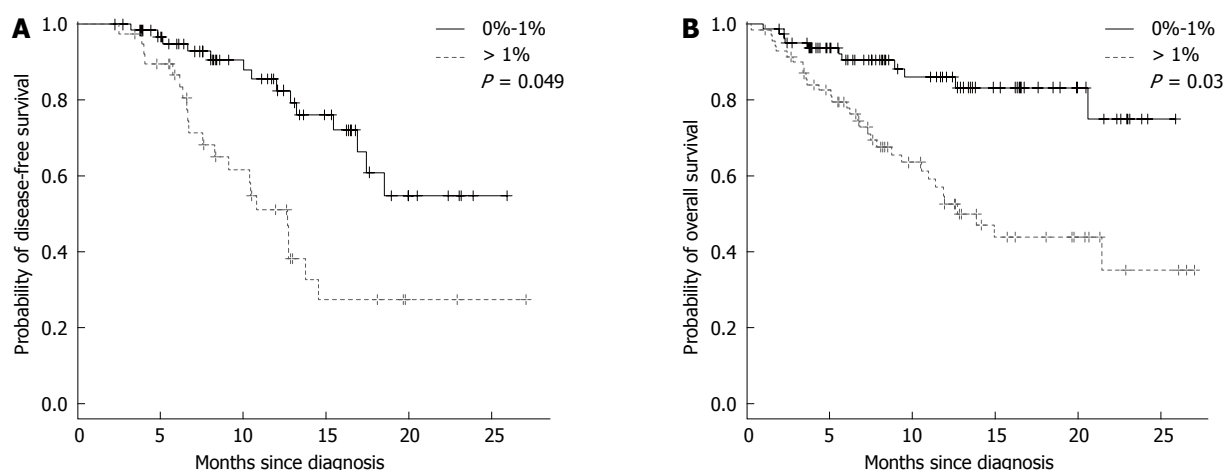


Figure 1 Survival of acute myeloid leukemia patients (without palliative) according to 1% CD34+CD38- cut-off. A: Disease free survival; B: Overall survival.

RESULTS

CD34+CD38- cell population in AML at diagnosis and correlation with other biological parameters

The proportion of CD34+CD38- immature leukemia cells was highly variable among the 200 adult and pediatric AML samples included in this series with a median value of 0.95% (range 0.01%-85.5%), with similar values in the adult (0.99%) and pediatric (0.5%) samples. In the adult patients, the proportion of CD34+CD38- immature leukemia cells was significantly correlated with age, FAB classification, cytogenetics and the expression of main molecular markers. The intensity of CD38 expression was significantly lower in patients older than 60 years than in younger patients (≤ 60 years) ($P < 0.001$, data not shown). When FAB classification was considered, we found a higher proportion of immature CD34+CD38- leukemia cells in M0 and M7 AML as compared to the other subtypes (Supplemental Figure 1A). The CD34+CD38- immature leukemic cell frequency was significantly higher in patients with unfavorable karyotypes than in those with favorable cytogenetics (excluding APL) or those with intermediate-risk cytogenetics ($P < 0.001$). The proportion of CD34+CD38- cells was significantly higher in the NPM1-FLT3ITD+ patient group than in the other groups but was not correlated with EVI1 status (Supplemental Tables 1 and 2).

In the favourable risk group, we observed a close correlation of % LSC (from total CD45/SSC blasts cells) with OS and quite significant for DFS, with longer survival for patients with lower level of CD34+CD38- LSC $< 1\%$ (median of OS and DFS not attempt $> 50\%$) compared with patients where %LSC was $> 1\%$ (median of OS 21.5 mo and for DFS 10.4 mo respectively), P -value = 0.0005 and 0.06. Interestingly, in the intermediate risk group, we observed the same significant correlation between the frequency of most immature CD34+CD38- blasts cells and survival: Median of OS and DFS 12.8 mo for patients with %LSC $> 1\%$ and not attempt for those with %LSC $< 1\%$; when only CD34+ compartment was analysed, we obtained similar results, a shorter OS

and DFS (median $> 50\%$ and 12.7 mo respectively) for %LSC $> 20\%$ compared with not attempt ($> 50\%$) for patients with %LSC $< 20\%$ (P -value = 0.004 and 0.009 respectively) (Data not showed).

CD34+CD38- LSC evolution between diagnosis and relapse

Among 109 patients in CR after induction chemotherapy, 33 relapsed (30%), with median time between diagnosis of 10 mo^[2-24], the majority from the group with higher frequency of LSC CD34+CD38- $> 1\%$ in blasts CD45/SSC (47%) compared with patients with LSC $< 1\%$ (20%) (P -value = 0.006). (Data not showed). Interestingly, there are no difference in terms of incidence of relapse between two groups when consider LSC% from CD34+ cells, suggested that the proportion of most immature stem cells from whole CD45 pool leukaemia cells is most predicted for aggressive clone than we regarded specifically CD34+ compartment, probably affected by residual normal HSC CD34+. There were significant differences between relapsed and no-relapsed patients in term of %CD34+CD38- as % from total blasts cells (median 1.53% vs 0.45% respectively) (P -value = 0.0098) but no difference when regarding %CD34+CD38- in CD34+ compartment (data not showed). The majority of patients that relapsed had unfavourable (13/33) or intermediate 2 (12/33) karyotype and 6/33 were in favourable group (3 patients with t(8;21) and 2 patients with inv16, and one had normal karyotype but AML1/ETO positivity) (Supplemental Table 3). Regarding molecular features, 4/33 relapsed patients had Evi1 overexpression, 4 patients FLT3ITD and 3 patients NPM1 mutation. Interestingly, most patients which relapsed had NPM1-/FLT3ITD- profile (26/33) with median of % LSC CD34+CD38- from CD45 total blasts cells $> 1\%$ [1.62% (0.04-56.8)]. Among 33 relapsed patients, 13 received allogeneic stem cell transplant, and 10 died for refractory disease.

Concerning favourable group, the frequency of CD34+CD38- cells was observed slightly higher at relapse compared at diagnosis for 4/6 patients suggested a negative influence of most immature CD34+CD38-

leukaemia cells level in these patients considered initially as favourable group.

We also evaluated the %CD34+CD38- frequency at relapse time for 26 patients with available immunophenotype data for both diagnosis and relapse time, and we compared with diagnosis level. We observed globally an increase of the most immature stem cell compartment at relapse in almost half of relapsed patients (12/26 patients; 46.15%) with accumulation of leukaemia blasts in CD34+CD38- compartment or eventually clonal selection of most immature LSC (median %LSC at relapse 2.16% vs 1.53% in diagnosis) but statistically not significantly (P -value = 0.2). Moreover, when compared intensity of CD38 level (express as ratio MFI of CD38/Isotype control) between diagnosis and relapsed paired samples we observed a significant higher expression at diagnosis total CD45/SSC blasts cells but also in CD34+ leukaemia subpopulation compared with relapse (median of rMFI CD38 in CD45/SSC blasts cells 25 vs 54 and 25.7 vs 66.7 in CD34+ compartment). The level of CD34+ expression in total blasts cells was significantly higher at relapse compared at diagnosis with median of 94% and 77% respectively (P -value = 0.001). These results suggest a continuous dynamic of LSC with clonal evolution and continuous selection in phenotype level of CD34+ leukaemia cells and most immature self-renewal LSC.

Concerning refractory or non-responders AML patients (55/163; 33.7%); we observed a significantly increase of median LSC CD34+CD38- levels in total CD45/SSC blasts cells > cut-off de 1% (median of 2.1%). The majority of these refractory/non-responder patients (34/55; 61.81%) presented at diagnosis with higher frequency of most immature CD34+/CD38-leukemia cells (> 1%) suggested again the major impact of these LSC population CD34+CD38- in mechanism of chemoresistance.

Correlation of CD34+CD38- population with patient outcome

Induction therapy achieved CR in 67% of cases, with a median follow-up of 7.6 mo (1-27.1) and a median time to relapse of 10.4 mo (1.9-27.1 mo). The CR rate was significantly higher in patients with a lower proportion of CD34+CD38- (< 1%) than in those with higher CD34+CD38- (> 1%) (79% vs 52%, P = 0.0005). The proportion of CD34+CD38- cells was significantly lower in patients achieving CR when compared to that of those who failed (0.53% vs 2% respectively, P = 0.0005). Similarly, a significant difference was observed among relapsing and non-relapsing patients regarding the percentage of immature CD34+CD38- leukemia cell observed at the time of diagnosis (Supplemental Figure 1B). A high percentage of CD34+CD38- was significantly associated with a shorter DFS (median DFS: 12.7 mo in patients with CD34+CD38- > 1% vs not reached in patients with CD34+CD38- < 1%) (Figure 1A) and a shorter OS (median OS: 14 mo in patients with CD34+CD38- > 1% vs not reached in patients with CD34+CD38- < 1%) (P = 0.03) (Figure 1B). In univariate analysis, a high

percentage of CD34+CD38- (> 1%) was correlated with a significantly shorter DFS (P < 0.0001) and OS (P = 0.0004) (Supplemental Table 4). In a multivariate analysis considering age, white blood cell count, percentage of CD34+ blasts and molecular characteristics, this factor appeared as an independent prognostic parameter for both DFS and OS (Table 1).

DISCUSSION

In this study, we confirmed that high stem cell frequency based on CD34/CD38 profile at diagnosis is a prognostic significance regarding to OS and disease-free survival (DFS). Our data are in agreement with data previously published by van Rhenen *et al.*^[9,10] showing a prognostic impact of the proportion of the CD34+CD38- stem cell population in 92 AML patients. Relapse of AML is thought to originate from resistant leukemic cells, residual cells at very few level as minimal residual disease (MRD), a higher CD34+CD38- population has no major impact on the CR rate and MRD after induction, but the most resistant fractions of the CD34+CD38- compartment seems to be selected with additional courses of chemotherapy. Keyhani *et al.*^[20] demonstrated that patients with AML showed a high CD38 intensity had significantly longer CR duration and survival compared with those with lower expression, suggesting that CD38 expression is a potentially useful independent marker of disease outcome. Several studies have demonstrated the prognostic value of CD34 expression in AML leukemic cells at diagnosis^[21], suggesting that CD34 expression is associated with lower CR rates and a shorter OS, but this has been not confirmed by all groups^[22]. In our series, we observed any significant correlations between CD34 expression and both OS and DFS in a multivariate analysis. Conversely, we observed a correlation between the percentage of CD34+CD38- leukemia cells and the total level of CD34+ blasts cells, more immature CD34+CD38- leukaemia cells correlating well with total level of CD34+ blasts cells, suggested anyway more aggressive potential of CD34 compartment when associated with CD38 analyse. These results emphasize the strong heterogeneity in CD34+ leukemia cells and the need for more detailed simultaneous analyses of CD38 combined with CD34 and quantification of the most immature CD34+CD38- stem cell compartment.

Interestingly, we observed a very high level of CD34+CD38- quantified in CD34+ compartment among the AML patients with leukaemia cells negative for CD34 (< 1%), previously considered as normal residual HSC in NPM1 mutated AML subtype^[5]. Anyway, reduced expression of CD38 in these CD34+ cells suggested a block in differentiation of the residual HSC. It was demonstrated recently^[23] that normal HSC in bone marrow from AML patients were more quiescent compared to HSC in normal healthy group, normal CD34+CD38- cells divided less when cultured with AML than alone, even when they are not in direct contact with HSC suggesting a soluble factor responsible for increased quiescence in normal

Table 1 Impact of prognostic factors on disease free survival and overall survival

Prognostic factors	Impact of prognostic factors on overall survival OS (wo palliatives, wo M3), <i>n</i> = 153				Impact of prognostic factors on disease free survival DFS (wo palliatives, wo M3), <i>n</i> = 101			
	Univariate analysis <i>P</i> value	Multivariate analysis <i>P</i> value	95%CI	HR	Univariate analysis <i>P</i> value	Multivariate analysis <i>P</i> value	95%CI	HR
Age > 60 yr	<i>P</i> < 0.0001	<i>P</i> = 0.02268	1.15-6.13	2.65	<i>P</i> = 0.21	<i>P</i> = 0.76834	0.33-2.25	0.87
WBC	<i>P</i> = 0.74	<i>P</i> = 0.10429	1-1.02	1.01	<i>P</i> = 0.13	<i>P</i> = 0.00184	1.01-1.02	1.01
Cytogenetic risk subgroup	<i>P</i> < 0.0001							
Favorable		<i>P</i> = 0.31675	0.43-13.33	2.4				
Intermediate		<i>P</i> = 0.05414	0.97-33.01	5.66				
Unfavorable								
Molecular anomalies								
FLT3ITD-					<i>P</i> = 0.82	<i>P</i> = 0.70619	0.32-5.27	1.31
NMP1-					<i>P</i> = 0.04	<i>P</i> = 0.01139	1.79-98.56	13.29
EVI1-					<i>P</i> = 0.89	<i>P</i> = 0.47958	0.38-8	1.74
%CD34 of Blasts	<i>P</i> = 0.47	<i>P</i> = 0.08765	1-1.03	1.01	<i>P</i> = 0.15	<i>P</i> = 0.95058	0.99-1.02	1
%CD34+CD38- of Blasts > 1%	<i>P</i> = 0.0001	<i>P</i> = 0.03091	1.09-6.43	2.65	<i>P</i> = 0.0005	<i>P</i> = 0.04663	1.02-7.73	2.8

This analysis excluded palliative cases and M3 subtypes. OS: Overall survival; DFS: Disease free survival; WBC: White blood cell.

residual HSC, the Taussig group trying to identify this factor. Whether this quiescent of normal residual HSC has possible impact in prognostic of AML CD34- patients should be confirmed in the large prospective studies.

Considering our understanding of leukemogenesis one of the most important questions arise from the cellular origin of the leukaemia stem cells. It was showed previously that AML-LSC were identified and purified in CD34+CD38- fraction cells between all bulk blast population in AML patients, and represented the only AML cells capable of self/renewal^[3,4,6]. Nonetheless, importantly heterogeneity has been revealed. Recent studies suggest that in some patients, AML-LSC could have a progenitor phenotype CD34+CD38+ and in patients with NPM mutation, LSC could be identified in the CD34- fraction^[5,7,8]. Recently more evidence on the heterogeneity of AML was described in terms of karyotype, differentiation stage of the blasts and clinical outcome, consequently it is remarkable that AML-LSC is more complex than previously thought, and can be different from patient to patient but also in the same patient regarding on the stage of disease with other mutations acquired in the original LSC that might occur during the development of the leukaemia, suggesting that LSC might represent a "moving target" even in the same patient.

Most recently, Goardon *et al.*^[12] investigated 74 primary human AML patient samples. It was showed that the CD34+ cells in about 80% of these cases contained two most frequent populations: One CD38-CD90-CD45RA+ (lymphoid-primed multipotential progenitor LMPP-like cells) and the other CD38+CD110+CD45RA+ (representing granulocyte-monocyte progenitor GMP-like cells). Both corresponded to normal hematopoietic progenitor populations rather than HSC and possess LSC activity based on their capacity of serially transplant the AML in immunodeficient NOD/SCID/IL2Rgamma null mice. Moreover, these populations showed a hierarchically organisation; whereby the CD38-CD45RA+

cells gave rise the GMP-like cells but not vice versa. Summary, these results enlarge on the previous view of AML LSC and settled a hierarchy of populations with decreasing frequency of LSC, implicate normal hematopoietic progenitors, LMPP and/or GMP as the cell of origin for AML/LSC in much of cases. One of the crucial implication of a progenitor phenotype profile for AML LSC connect to the critical stem cell property of self-renewal, the acquisition of self-renewal ability in AML LSC being consider as an aberrant event resulting from genetic and/or epigenetic features. Thereafter, the most significant incrimination of the leukaemia stem cell model is that to eradicate the leukaemia and cure the patient. The final goal could be eradication of all LSC, the analyses of gene expression data reported by Goardon in this study would be one of the most important step toward the identification of these LSC specific genes or pathways.

Taken together, the results of our study emphasis that using CD34/CD38 "backbone" in leukaemia cells analysis by multicolour flow cytometry at AML samples at diagnosis is relatively facile method, rapidly translate in clinical practice as complementary prognostic factor in AML. Multicolour/multidimensional flow cytometry represent very useful tools to identify and characterise immunologic profile of different leukaemia compartments using CD34/CD38/CD45 as "backbone" to design more complex panels (8-10-14 colours) adapted to AML diagnosis and MRD flow evaluation, including most specific LSC markers described previously as CLL-1, TIM3, CD123, CD45RA, CD97, CD47, CD44, CD49f, to better discriminate between nHSC and LSC as described recently^[24,25]. The origin of AML LSC is still controversy, however all fundamental experience to elucidate the function of stem cells are based to first in CD34/CD38 selected populations subsequent transplanted to immunodeficient mice, suggested the importance of CD34/CD38 analyse of all AML leukaemia cell at diagnosis and relapse and quantification of most immature "profile"

CD34+CD38- in all "bulk" leukaemia blasts population as basically immunophenotype that should be used in all leukaemia immunological evaluation, associated with others lineage or stem cells markers.

In summary, we showed that the presence of a CD34+CD38- subpopulation representing more than 1% of total "bulk leukemic cells" at diagnosis could help to identify patients at risk of induction failure and poor outcome. This method is simple, rapid and accurate and can easily be applied to the clinical practice. Despite heterogeneity and complexity of AML LSC we could still use CD34+CD38- to predict patient outcome and it is still a potential tool.

These results should be confirmed in a prospectively larger cohort of patients and could be considered as useful complementary prognostic parameter for risk-stratification AML patients in future clinical trials.

COMMENTS

Background

Multicolor flow cytometry is largely used for acute myeloid leukemia (AML) diagnosis in most of Hematology departments for lineage assessment based on ELN and WHO 2016 guidelines. Quantification of most immature CD34+CD38- leukemia blast cells could be easily included at diagnosis panel, this method being simple, rapid and accurate and be applied to the clinical practice.

Research frontiers

CD34+ compartment the authors divided three subpopulations: CD34+CD38-, CD34+CD38lo, and CD34+CD38hi, based on intensity of CD38 expression; FMO (Fluorescence Minus One) was used for CD38- level, and hematogones populations for CD38hi level. The stem cell compartment CD34+CD38- contain very few events in some patients but these events should tightly cluster in a FSC/SSC plot and CD45/SSC plot. The authors evaluated also the intensity of fluorescence signal for CD38 quantified as rMFICD38 from CD34+ gated cells and rMFICD38 from CD45lo/SSC total blasts cells.

Innovations and breakthroughs

In this study, the authors confirmed that high stem cell frequency based on CD34/CD38 profile at diagnosis is a prognostic significance regarding to overall survival (OS) and disease-free survival (DFS). Relapse of AML is thought to originate from resistant leukemic cells, residual cells at very few level as minimal residual disease (MRD), a higher CD34+CD38- population has no major impact on the CR rate and MRD after induction, but the most resistant fractions of the CD34+CD38- compartment seems to be selected with additional courses of chemotherapy. The results emphasized the strong heterogeneity in CD34+ leukemia cells and the need for more detailed simultaneous analyses of CD38 combined with CD34 and quantification of the most immature CD34+CD38- stem cell compartment.

Applications

Multicolour/multidimensional flow cytometry represent very useful tools to identify and characterise immunologic profile of different leukaemia compartments using CD34/CD38/CD45 as "backbone" to design more complex panels (8-10-14 colours) adapted to AML diagnosis and MRD flow evaluation, including most specific LSC markers described previously as CLL-1, TIM3, CD123, CD45RA, CD97, CD47, CD44, CD49f, to better discriminate between nHSC and LSC.

Terminology

Leukemia initiating cells (LICs) or Leukemia stem cells (LSC) in mice have shown a primitive immunophenotype (CD34+CD38-) with similarities to normal hematopoietic stem cells (HSCs) regardless of the subtype of AML or the immunophenotype of the majority of the leukemic blasts present in the bone marrow. However, other studies showed that LSC were exclusively found in the

CD34- compartment. Other investigators described LSC in both the CD34- and CD34+ cell compartments.

Peer-review

The study is well-designed and its topic is interesting.

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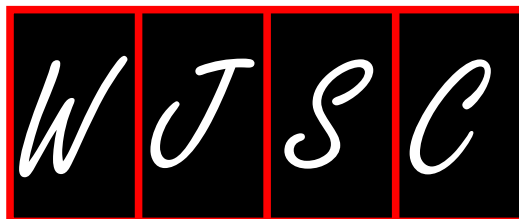
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Umbilical cord blood stem cell treatment for a patient with psoriatic arthritis

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Abstract

Clinical and laboratory results document psoriatic arthritis in a 56-year old patient. The symptoms did not resolve with standard treatments (nonsteroidal anti-inflammatory drugs, steroids and methotrexate). TNF-alpha inhibitors (certolizumab pegol and adalimumab) were added to the treatment regime, with some adverse effects. A trial of human umbilical cord stem cell therapy was then initiated. The stem cells were enriched and concentrated from whole cord blood, by removal of erythrocytes and centrifugation. The patient received several infusions of cord blood stem cells, through intravenous and intra-articular injections. These stem cell treatments correlated with remission of symptoms (joint pain and psoriatic plaques) and normalized serologic results for the inflammatory markers C-reactive protein and erythrocyte sedimentation rate. These improvements were noted within the first thirty days post-treatment, and were sustained for more than one year. The results of this trial suggest that cord blood stem cells may have important therapeutic value for patients with psoriatic arthritis, particularly for those who cannot tolerate standard treatments.

Key words: Umbilical cord blood stem cells; Stem cells; Psoriatic arthritis; Rheumatoid arthritis

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Core tip: Rheumatic diseases are common and often disabling. Standard drug treatments can control inflammation, but many patients do not find relief. Potent biologic drugs (tumor necrosis factor inhibitors) are not tolerated by some. This patient report describes treatment with umbilical cord blood stem cells (CBSC). Clinical observations and serology results document prolonged

improvement of psoriatic arthritis. This type of report is important since it documents a dosage, time course, and a beneficial outcome, using an under-employed type of stem cell. These results can help guide clinicians in future trials using CBSC.

Coutts M, Soriano R, Naidoo R, Torfi H. Umbilical cord blood stem cell treatment for a patient with psoriatic arthritis. *World J Stem Cells* 2017; 9(12): 235-240 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v9/i12/235.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v9.i12.235>

INTRODUCTION

Psoriatic arthritis (PsA) is a chronic inflammatory disease that can result in significant disability. It is characterized by skin rashes, fatigue, and swollen, painful joints. The disease can be progressive; about 20% of the patients develop a severe form of the disease with multiple joint deformities and bone erosion^[1,2]. The inflammation associated with PsA can lead to damage in a variety of tissues: the GI tract, heart, lungs, liver and kidney. Remission of symptoms and a better prognosis are more likely when treatment begins early^[3,4].

PsA and rheumatoid arthritis (RA) can be difficult to distinguish. Both are autoimmune syndromes where the joints are attacked. The same sets of joints can be affected with tenderness and swelling, though certain patterns tend to be indicative of PsA or RA. There can be similar serology (elevated levels of C-reactive protein and increased erythroid sedimentation rate). These tests measure systemic inflammation, and are not specific for PsA nor RA. To further complicate diagnosis, the onset of psoriatic plaques in PsA can vary. For example, psoriasis precedes PsA in 70%-80% of the patients, while arthritis precedes psoriasis in 15%-20% of the patients. In the remainder of the patients, onset of both symptoms is within a year^[5]. In the pre-symptomatic stages of these diseases, inflammation starts at the entheses in PsA, while inflammation starts in the synovium in RA. However, both diseases can progress to synovitis and bone erosion^[5,6]. PsA and RA can often be differentiated by MRI; PsA also has a distinct synovial membrane vascularity and an over-representation of the Th17 subset of T cells^[7]. However, these more definitive tests would not be used in an initial diagnosis.

Despite clinical and pathogenic differences, PsA and RA are often treated with the same drugs. Commonly prescribed medications include: Nonsteroidal anti-inflammatory drugs (NSAIDs), steroids, antirheumatic drugs such as methotrexate, and biologic anti-inflammatory drugs. This latter group includes adalimumab (Humira), and certolizumab pegol (Cimzia). Both biologics are antibodies that block the activity of tumor necrosis factor alpha (TNF). All of these medications can control symptoms and prevent joint damage. However,

in some patients, these drugs are ineffective or poorly-tolerated. The TNF inhibitors also have significant safety considerations, such as increased risk of opportunistic infections.

In this study, a subset of human umbilical cord blood cells was tested as an experimental treatment for PsA. Cord blood is readily available; its regenerative capabilities are being actively investigated for a number of clinical applications^[8,9]. Historically, cord blood and cord blood stem cells have been used for the treatment of hematopoietic disorders; therapies have been expanded to include immune modulation^[10]. Umbilical cord blood contains different types of stem cells, which may exert therapeutic and regenerative effects through a variety of mechanisms^[10,11]. Given the reports of regenerative and immunomodulatory effects, it is a logical step to test CBSC for inflammatory conditions like PsA.

CASE REPORT

The patient was a 56-year-old male with a two-year history of arthritic symptoms. He presented with joint stiffness and pain, particularly in the metacarpal and proximal phalangeal joints. The joint stiffness and pain was more pronounced upon waking. Six months after initial presentation, the pain and stiffness progressed to wrists, shoulders and jaw. Notably, the pain did not affect all areas simultaneously. The patient described the pain as "jumping" from region to region. Joint swelling was noted, particularly in the proximal interphalangeal and metacarpal joints. Some asymmetry was noted. The fingers of both hands had some degree of dactylitis; the fingernails were pitted and discolored. Tendons were inflamed, but structural deformities of the joints were not noted. Given the absence of psoriatic plaques, laboratory tests were ordered to evaluate the possibility of rheumatoid arthritis and gauge the level of inflammation. The Vectra DA test evaluates 12 serum proteins linked to RA disease activity; the test was scored with a "high level of RA disease activity" (score of 51, with < 1 as a reference value). Both anti-cyclic citrullinated peptide values and C-reactive protein levels were abnormally high (Anti-CCP > 1000, reference ≤ 20 U/mL, C-reactive protein (CRP) 5 to 15, reference range of < 1 mg/mL). The erythroid sedimentation rate (ESR) and values for Rheumatoid Factor were also elevated (ESR, 45, reference = 0-15 mm/h, RhF = 630, reference value 0 IU/mL). A time course of the patient's lab results, symptoms and treatments are presented in Figure 1.

The patient was initially treated with nonsteroidal anti-inflammatory drugs (NSAIDs) and methotrexate, but experienced minimal improvement. A synthetic corticosteroid (prednisone) was prescribed on an increasing dosing schedule. After one and a half years of these treatments, there were no significant improvements in arthritic symptoms. The patient was counseled to try certolizumab pegol, a TNF- α inhibitor. Since patients are more susceptible to opportunistic infections during anti-

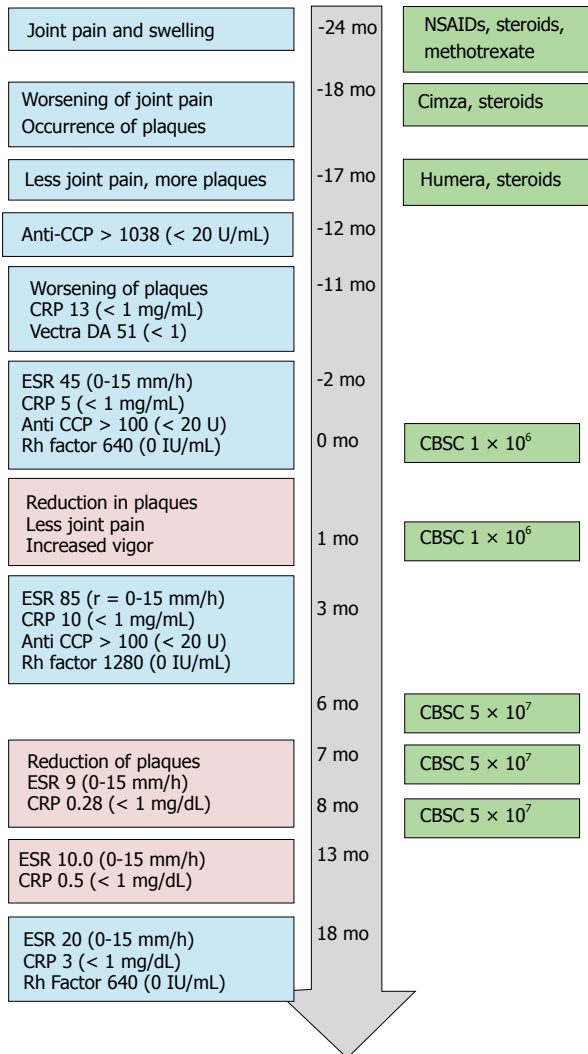


Figure 1 Time course and patient treatments. Time course as grey arrow [in months, first cord blood stem cells (CBSC) treatment starts month 0]. Green panels on right describe drug and CBSC treatments. Panels on left describe patient symptoms and lab results. Normal adult reference values for lab results are in parenthesis. Atypical results with blue background, normal results with beige background. NSAID: Nonsteroidal anti-inflammatory drugs; CBSC: Cord blood stem cells; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein.

TNF therapy, the patient was pre-tested for hepatitis, HIV, and tuberculosis and was found to be negative.

Within two weeks of certolizumab pegol injection, joint symptoms worsened and dermal psoriatic plaques occurred on the trunk and legs. The psoriatic plaques became open weeping wounds on the upper extremities, the trunk, and the lower extremities (Figure 2). Certolizumab was discontinued and another TNF- α inhibitor (adalimumab, brand name Humira) was used, instead. Prednisone treatment was continued. Adalimumab and prednisone improved joint symptoms, but psoriatic plaques worsened over the next 4-6 mo.

The patient sought out alternative therapies and underwent two series of intravenous injections of umbilical cord blood-derived cells in a Republic of Panama-based stem cell clinic. The injections took place one month apart; 200000 cells were administered on four or five consecutive days (for a total of 8×10^5 or 1×10^6 cells).

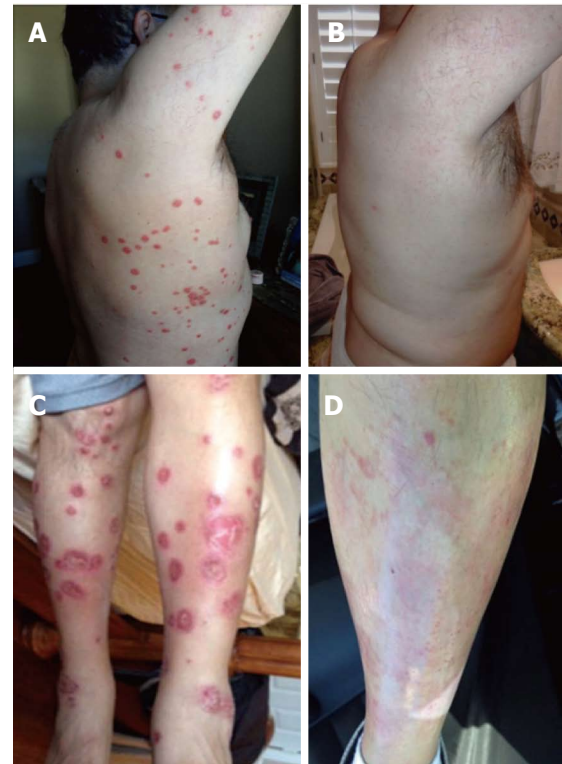


Figure 2 Images of patient trunk and legs, 11 mo prior to the first stem cell injection (A, C); Images of trunk and legs, 7 mo after the first cord blood stem cells infusion (B, D).

After one week, the patient noted some improvement of the psoriatic plaques, and reduction in joint pain. Two months after these initial cord blood stem cell injections, laboratory tests were repeated. Markers of inflammation increased: Erythrocyte sedimentation rate (ESR) was 85, CRP levels had doubled to 10.3 mg/L. Rheumatoid factor had also doubled (to 1280). Anti-CCP remained the same (> 100 U/mL, compared to a normal value of < 20 U).

Notwithstanding the lack of improvement in laboratory data, the patient was encouraged by the reduction in the number and size of psoriatic plaques and his improved sense of vitality. He sought out treatments of cord blood stem cells in the United States and had three rounds of injections, six months after the initial stem cell injections in Panama. The physician in the US-based clinic chose a method of treatment that included a fifty-fold greater number of nucleated umbilical cord blood stem cells. About 5×10^7 CBSC were injected intravenously and intra-articularly, once a month for three months (Figures 1 and 3). The patient reported gradual improvement of associated joint pain and swelling, "higher energy levels" and increased physical capabilities.

Psoriatic plaques were greatly resolved after the third treatment with CBSC (Figures 1 and 2). The psoriatic plaques were almost completely absent from the trunk and limbs. The few that remained were dramatically reduced in size, less raised and less red, and no longer scaly. Significantly, the inflammatory markers of ESR and CRP were within normal range after the fourth round of CBSC injections. The erythrocyte sedimentation

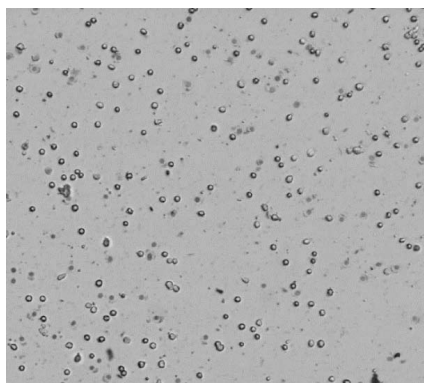


Figure 3 Umbilical cord blood stem cells, magnification approximately 150 x. Sample image by automated cell counter, BioRad TC20.

rate (ESR) was measured as 9 mm/h, and the CRP value was 0.28 mg/dL). Five months after the last CBSC injection, ESR and CRP levels remained normal. Eighteen months after the initial CBSC injection, ESR and CRP levels had increased slightly out of normal range, but were far below levels reported prior to CBSC treatment. A reduced dosage of prednisone was continued, during and after CBSC treatment. The patient did not report any significant lifestyle changes over the course of the study (no recreational drugs, smoking, very limited use of alcohol). There were no serious complications related to CBSC treatment. There was mild sequela 48 h after the fifth and final injection; the patient had flu-like symptoms for two days (a mild febrile response and general soreness in muscles and joints). Lymphocyte counts were normal throughout the study. There was increased tolerance to exercise, as inflammatory arthritic symptoms decreased.

DISCUSSION

This patient report demonstrates several important aspects in treating inflammatory arthritis: Difficulties in diagnosis, the failure of some patients to achieve relief, and the need for new treatment modalities. This patient was initially diagnosed with RA. Definitive diagnosis of PsA or RA can be difficult given the heterogeneity of the diseases and the lack of definitive serology markers. Certain autoantibodies are considered diagnostic of RA: Rheumatoid factor (Rh factor) and cyclic citrullinated peptide antibody (Anti-CCP). However, 5%-13% of PsA patients are seropositive for these, and about 20% of RA patients are negative. ESR and CRP are widely used to guide anti-inflammatory treatments in RA, but are elevated in only about half of the patients with PsA. It should be noted that elevated ESR (> 15 mm/h) can be a very useful marker in treating PsA, since high levels are associated with a more severe disease state and increased mortality^[12]. Ultimately, the patient received a diagnosis of PsA since the skin lesions, nail pitting, asymmetric joint pain and general dactylitis (vs joint-specific swelling) are consistent with PsA, and the serology report is indeterminate for a differential diagnosis.

Both PsA and RA treatment regimens usually follow a "step-up" approach. Treatments are tailored to the specific patient, different classes of drugs assessed, evaluations repeated over time and therapies altered, as appropriate. The main principals of treating both rheumatic illnesses are the same: Controlling symptoms and preventing damage, improving the quality of life for the patient, and minimizing side effects^[13,14].

This patient's symptoms were refractory to standard treatments of steroids and methotrexate. Adding a biologic to the treatment regime is standard of care; the TNF inhibitors certolizumab and adalimumab have similar beneficial results (52%-58% of PsA patients, depending on dosage)^[4]. However, in this case, administration of certolizumab correlated with increased joint pain and the appearance of psoriatic plaques. Joint symptoms improved somewhat with the substitution of adalimumab, but the psoriatic plaques worsened. Switching from one TNF inhibitor to another is helpful for 30%-74% of the PsA primary non-responders (percentage depending on initial and secondary drug), but this leaves a significant cohort that respond to neither. Further, 20%-71% of patients administered TNF inhibitors reported treatment-emergent adverse events^[15]. In addition to the risk of serious side effects, TNF inhibitors may lose efficacy or fail, due to the patients developing antibodies against them^[16]. This patient did not have satisfactory improvement after treatment with NSAIDs, steroids, methotrexate and TNF inhibitors; in addition to experiencing adverse effects that correlated with treatment.

This case may have an unusual presentation for PsA, but it highlights a problem common to all inflammatory arthropathies. Some patients will progress to severe joint damage and experience significant morbidity or even loss of life. Others will be only mildly affected, and not progress to irreversible joint damage. A prognostic test or biomarker has not been developed, so we can't identify the patients who would benefit from early and aggressive treatments. At the same time, it is important not to over-treat patients. Many, left untreated, would not progress to joint destruction. The potential side effects and high costs of anti-TNF biologicals make this point especially salient.

Hence, there is a need to seek out new therapies for inflammatory arthritic diseases. Stem cells, and specifically cord blood stem cells, have been used for regenerative therapies and immune modulation^[10,11]. The mesenchymal stem cells (MSC) or MSC progenitors, present in umbilical cord blood are of particular interest as effectors, since they release a variety of regenerative growth factors such as VEGF, FGF, and PDGF^[17]. Interestingly, MSC can migrate to sites of injury and inflammation and have the potential to "calm down" an overactive immune system^[18]. This observation is the basis of experimental MSC treatments for a variety of autoimmune diseases (e.g., type I diabetes, Crohn's disease, lupus, and rheumatoid arthritis). Preclinical data suggests that umbilical cord MSC may attenuate arthritic

diseases by increasing regulatory T cells while decreasing T follicular helper cells, in addition to decreasing the pro-inflammatory Th17 cells^[19]. Numerous reports conclude that the stem cells present in cord blood can support tissue repair and reduce excessive inflammatory responses through a variety of mechanisms^[8].

We cannot assign causality between the CBSC injections and this patient's clinical improvements, but the correlations are intriguing. The patient reported small, gradual improvements almost immediately after initial, small doses of CBSC injections (approximately 1×10^6 cells). After several months and higher numbers of CBSC (approximately 5×10^7), serological markers of inflammation fell into normal range and there were dramatic improvements in the number, size, color and consistency of the psoriatic plaques. In part, we cannot draw conclusions because we can't substantiate the quality and quantity of the cells administered in a foreign clinic. Another part is there is little or no published evidence from clinical trials using CBSC for inflammatory arthritis. As more studies are published, we will gain an understanding of dosage, an expected time course and anticipated results. The overall results from this patient suggest further clinical trials with CBSC should be pursued. He experienced a remarkable reduction in symptoms over an extended period of time (> 12 mo after the initial CBSC injections). It is hoped that this patient report will lead to additional studies using CBSC and ultimately give new treatment options to other RA and PsA patients.

ARTICLE HIGHLIGHTS

Case characteristics

A 56-year-old male patient presented with joint stiffness and pain, skin lesions and fatigue.

Clinical diagnosis

Swelling, pain and reduced range of motion in the metacarpal and proximal phalangeal joints; joint symptoms progressed to include wrist and shoulder joints. Stiffness and pain were more pronounced on waking. Two years of arthritic symptoms preceded red, scaly skin lesions.

Differential diagnosis

Rheumatoid arthritis, osteoarthritis, rheumatic fever, systemic lupus erythematosus, gout, secondary syphilis.

Laboratory diagnosis

Values for C-reactive protein (CRP) and red blood cell sedimentation rate (ESR) became normal after cord blood stem cell (CBSC) treatment.

Pathological diagnosis

Anatomical pathology indicating psoriatic arthritis (PsA) resolved after treatment with CBSC.

Treatment

Nonsteroidal anti-inflammatory drugs (NSAIDs), prednisone (steroid), methotrexate, TNF blockers (certolizumab and adalimumab), and umbilical cord blood stem cells.

Related reports

Mesenchymal stem cells (from bone marrow or fat) have been used to treat rheumatoid arthritis.

Term explanation

Human umbilical cord blood was obtained from CorCell Cord Blood. Erythrocytes were removed using an ammonium chloride lysis buffer, used according to manufacturer's directions (eBioScience). Flow analysis of similar preparations revealed about 3% of the population had the MSC marker, CD90⁺. Live, nucleated cells were concentrated by centrifugation, enumerated, and resuspended in cryopreservative (CryoGold Serum-Free Freezing Media, purchased from Stemgent, Inc.) The resulting samples were aliquoted into 1.8 mL cryogenic vials and gradually chilled to -160°C . Immediately before use, cells were thawed and assessed for viability. Post thaw viability was 80%, as determined by an automated cell counter (Bio-Rad TC-20). Figure 3 shows one of the preparations administered to the patient.

Experiences and lessons

This patient had persistent PsA symptoms, and did not find relief with standard therapies. Remission of symptoms correlated with injections of umbilical cord blood stem cells. Serological tests for inflammation (ESR and CRP) were normal for over a year after the initial CBSC injections.

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