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REVIEW

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

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 raise 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. Wangensteen *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 neurorrhaphy 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-κB 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 s 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 PCl2 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 remotion 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 PCl2 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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MINIREVIEWS

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

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), NT5E (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 heterogenous 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-κB) 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-κB), 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-κB 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-β1 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 (PPARγ and CEBPβ)[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 PPARγ 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-β1/SMAD4) and SMAD-independent (*e.g.*, TGF-β1/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

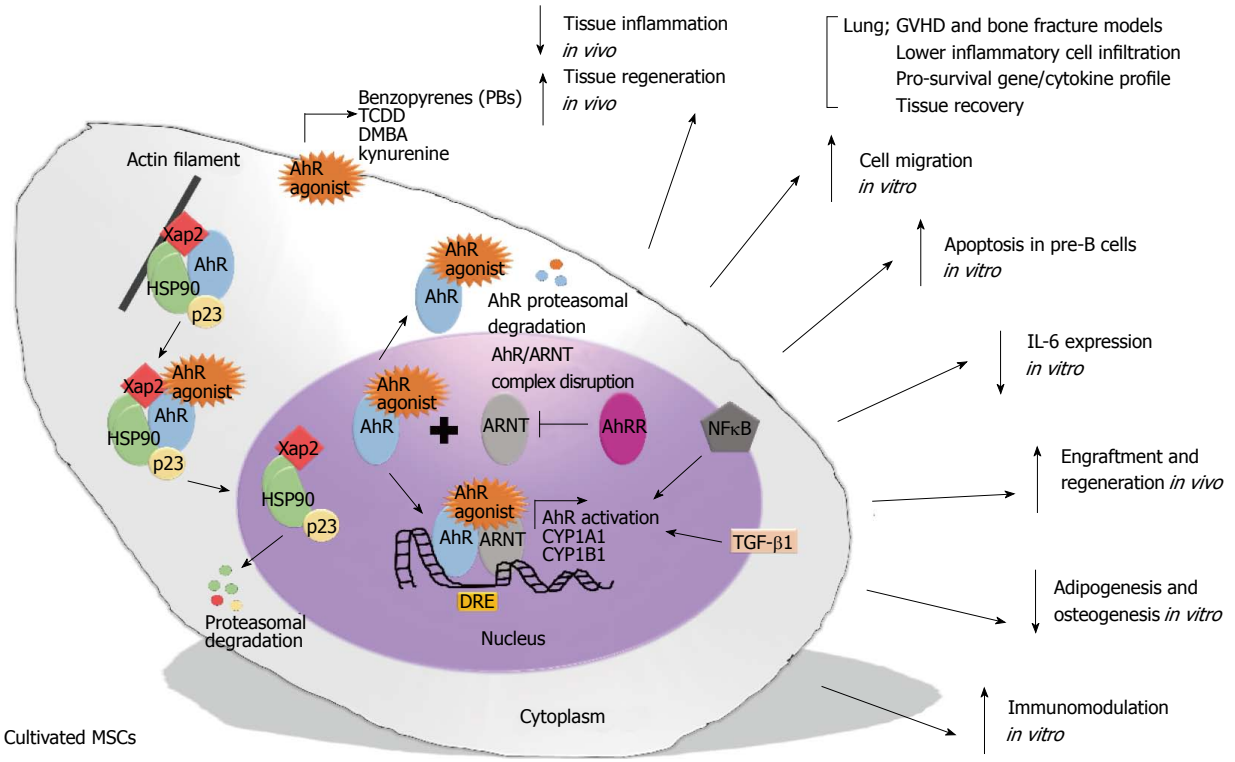

Figure 1 Illustration demonstrating a hypothetical summary of the potential effect of aryl-hydrocarbon receptor activation on multipotent mesenchymal stromal cell function. AhR-mediated MSC activation occurs by a cascade of events that substantially modulate the function of the MSCs by mechanisms associated 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.*, IFNγ, 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 (IFNγ 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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ORIGINAL ARTICLE

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 *Snai*, *Zeb* 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

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.

Jayachandran A, Shrestha R, Dhungel B, Huang IT, Vasconcelos MYK, Morrison BJ, Ramlogan-Steel CA, Steel JC. Murine hepatocellular carcinoma derived stem cells reveal epithelial-to-mesenchymal plasticity. *World J Stem Cells* 2017; 9(9): 159-168 Available from: URL: http://www.wjgnet.com/1948-0210/full/v9/i9/159.htm DOI: http://dx.doi.org/10.4252/wjsc.v9.i9.159

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 ℃ under a humidified atmosphere with 5% CO_2 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 ℃ in a humidified atmosphere of 5% CO_2 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 ℃-30 ℃) 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 synthesis kit (Bioline, Australia).

Table 1 List of primers for quantitative reverse transcriptase-PCR

Primer	Sequence (5′-3′)
ActB forward	ATGGAGGGGAATACAGCCC
ActB reverse	TTCTTTGCAGCTCCTTCGTT
Klf4 forward	CAGTGGTAAGGTTTCTCGCC
Klf4 reverse	GCCACCCACACTTGTGACTA
Bmi1 forward	TGGTTGTTCGATGCATTTCT
Bmi1 reverse	CTTTCATTGTCTTTTCCGCC
Cd44 forward	AGCGGCAGGTTACATTCAAA
Cd44 reverse	CAAGTTTTGGTGGCACACAG
E-Cadherin forward	AAAAGAAGGCTGTCCTTGGC
E-Cadherin reverse	GAGGTCTACACCTTCCCGGT
ZO-1 forward	CCTGTGAAGCGTCACTGTGT
ZO-1 reverse	CGCGGAGAGAGACAAGATGT
Vimentin forward	AGAGAGAGGAAGCCGAAAGC
Vimentin reverse	TCCACTTTCCGTTCAAGGTC
Fibronectin forward	ACTGGATGGGGTGGGAAT
Fibronectin reverse	GGAGTGGCACTGTCAACCTC
Snai1 forward	AGTGGGAGCAGGAGAATGG
Snai1 reverse	CTTGTGTCTGCACGACCTGT
Snai2 forward	GATGTGCCCTCAGGTTTGAT
Snai2 reverse	GGCTGCTTCAAGGACACATT
Zeb1 forward	TCATCGGAATCTGAATTTGC
Zeb1 reverse	CCAGGTGTAAGCGCAGAAAG
Zeb2 forward	TGCGTCCACTACGTTGTCAT
Zeb2 reverse	TCTTATCAATGAAGCAGCCG
Twist1 forward	CATGTCCGCGTCCCACTA
Twist1 reverse	TCCATTTTCTCCTTCTCTGGA
Twist2 forward	GCCTGAGATGTGCAGGTG
Twist2 reverse	GTCTCAGCTACGCCTTCTCC

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 ℃ for 5 s, annealing at 60 ℃ for 10 s and extension at 75 ℃ 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 ℃ for 10 s and combined annealing/extension at 60 ℃ 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*-CTtarget) × 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'

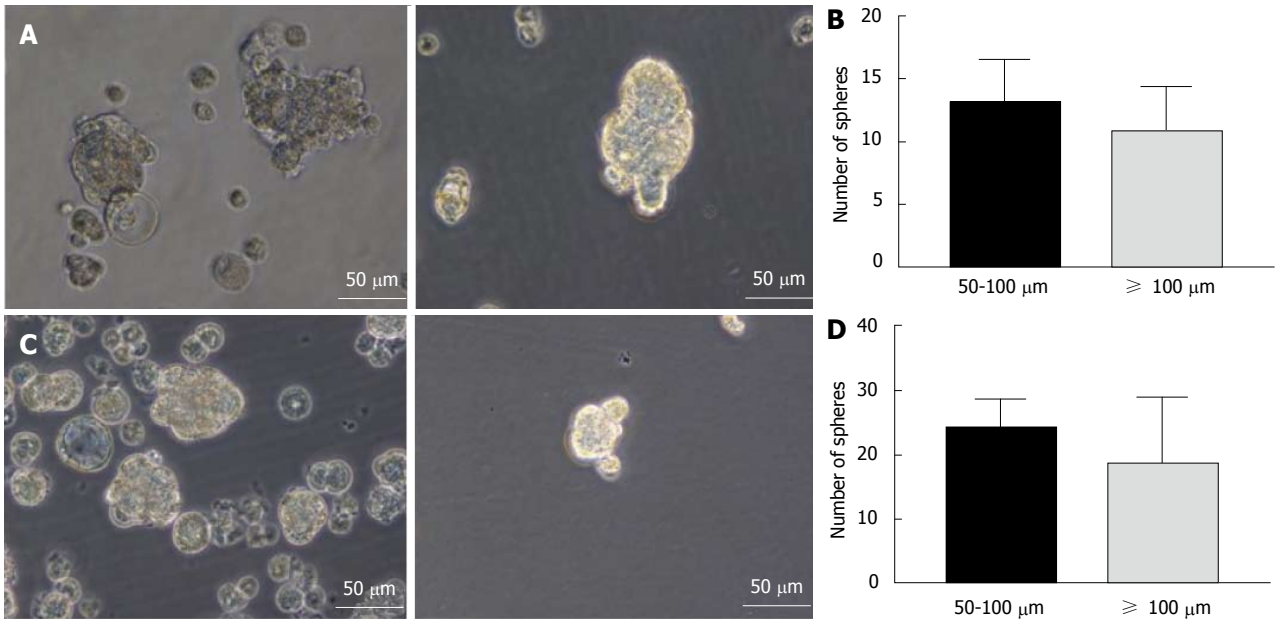

Figure 1 Enrichment of cancer stem cells using the sphere culture method. A: Photomicrographs of Hepa 1-6 3-dimensional spheres on day 5 cultured in stem cell conditioned serum-free medium; B: Hepa 1-6 sphere sizes and numbers were enumerated; C: Photomicrographs of AML12 3-dimensional spheres on day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

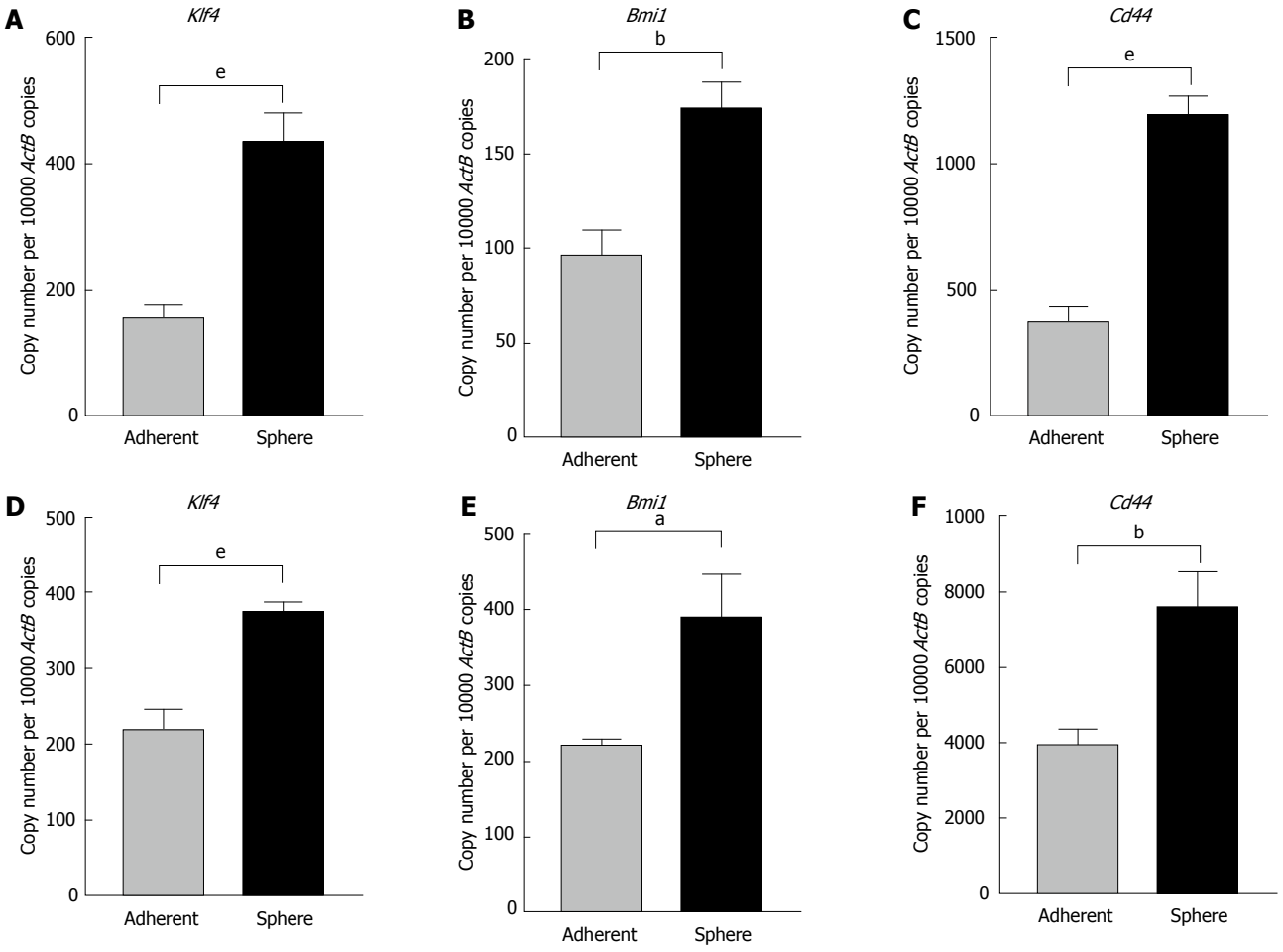

Figure 2 Three-dimensional spheres expressed high stemness and cell surface cancer stem cell markers. qRT-PCR analysis revealed higher expression of A: *Klf4*, B: *Bmi1* and C: *Cd44* in Hepa 1-6 3-dimensional spheres compared with Hepa 1-6 grown as adherent cells. qRT-PCR analysis revealed higher expression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

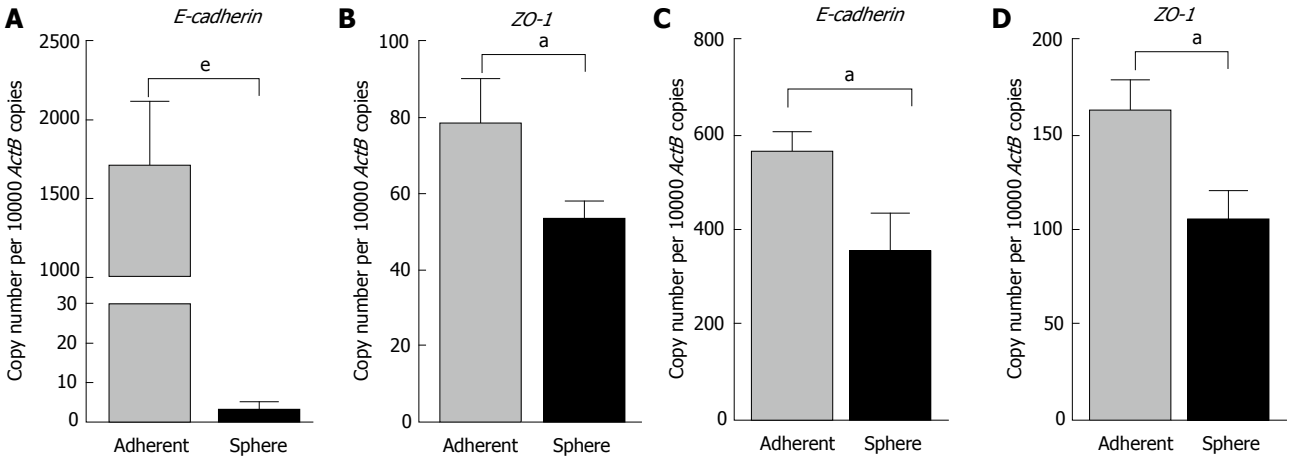

Figure 3 3-dimensional spheres decreased expression of epithelial markers. qRT-PCR analysis revealed downregulation of A: *E-cadherin* and B: *ZO-1* in Hepa 1-6 CSCs compared with Hepa 1-6 grown as adherent cells. qRT-PCR analysis revealed lower expression of C: *E-cadherin* and D: *ZO-1* in AML12 CSCs compared with AML12 grown as adherent cells. Values are mean ± SEM of three experiments in triplicate ($^{a}P < 0.05$). 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

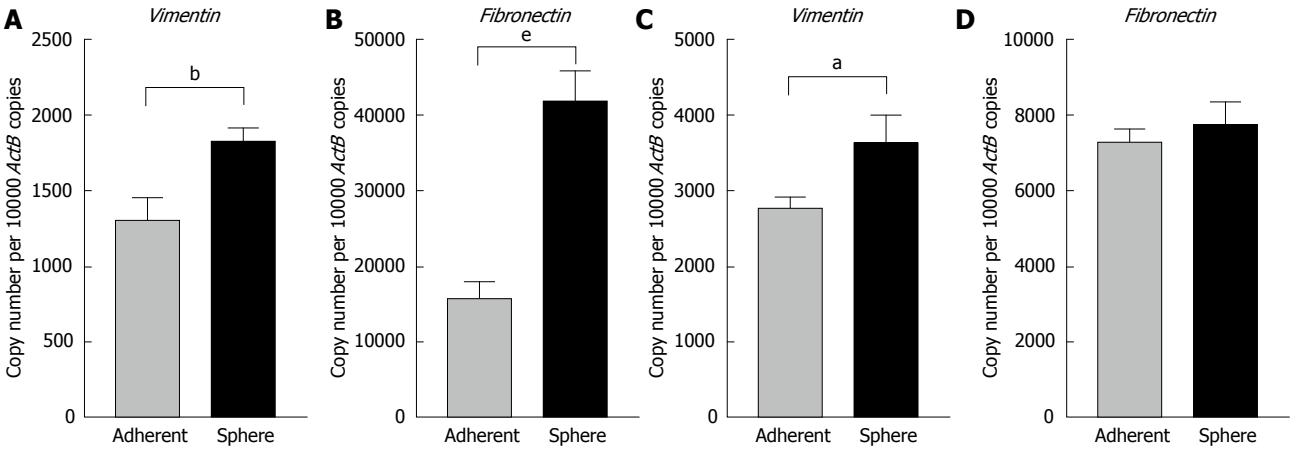

Figure 4 Three-dimensional spheres have higher expression of mesenchymal markers. qRT-PCR analysis revealed upregulation of A: *Vimentin* and B: *Fibronectin* in Hepa 1-6 3-dimensional spheres compared with Hepa 1-6 grown as adherent cells. qRT-PCR analysis revealed higher expression of C: *Vimentin* and D: *Fibronectin* in AML12 3-dimensional spheres compared with AML12 grown as adherent cells. Values are mean ± SEM of three experiments in triplicate ($^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$). 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 *Twist*2 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

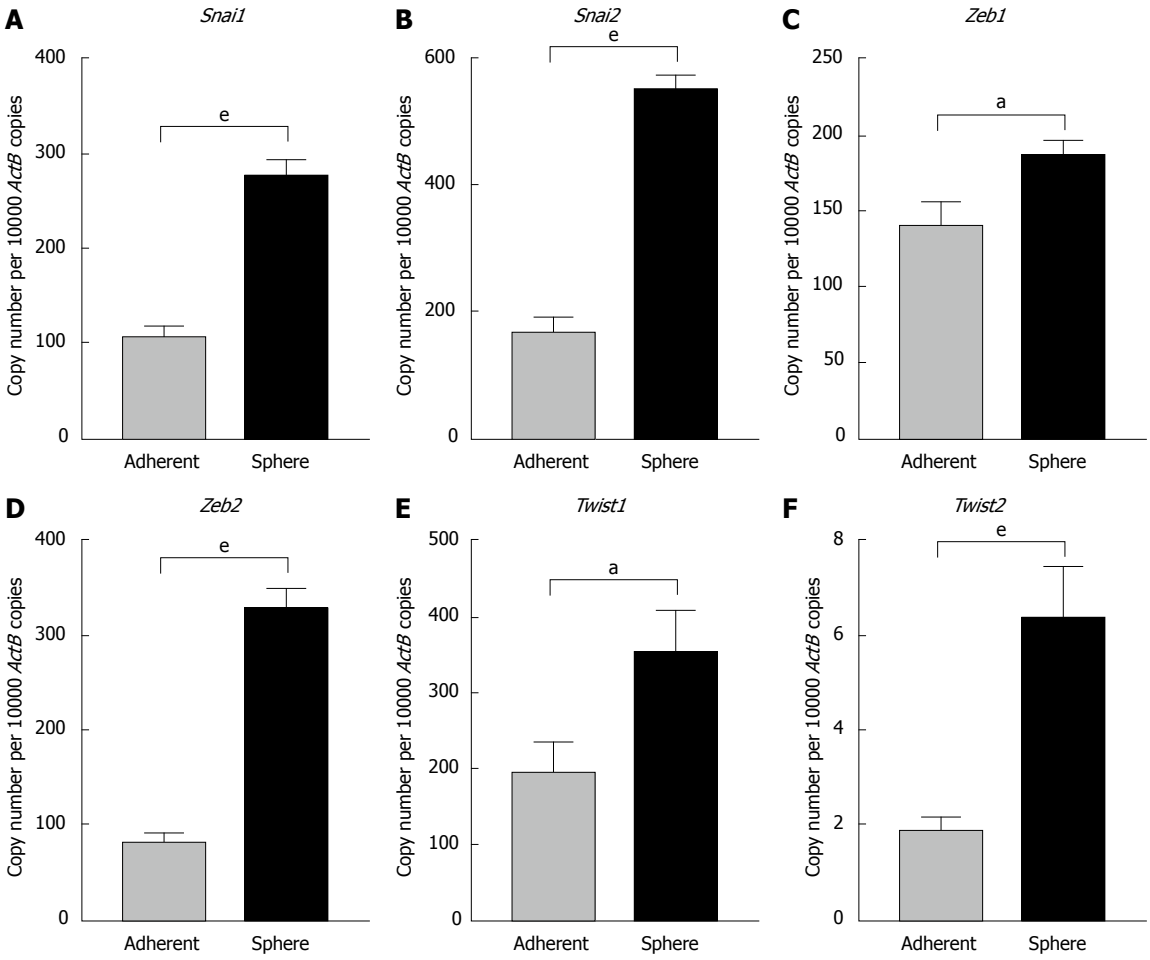

Figure 5 Expression of putative epithelial-to-mesenchymal transition transcription factors in Hepa 1-6 3-dimensional spheres. qRT-PCR analysis revealed upregulation of A: *Snai1*, B: *Snai2*, C: *Zeb1*, D: *Zeb2*, E: *Twist1*, and F: *Twist2* in Hepa 1-6 3-dimensional spheres compared with Hepa 1-6 grown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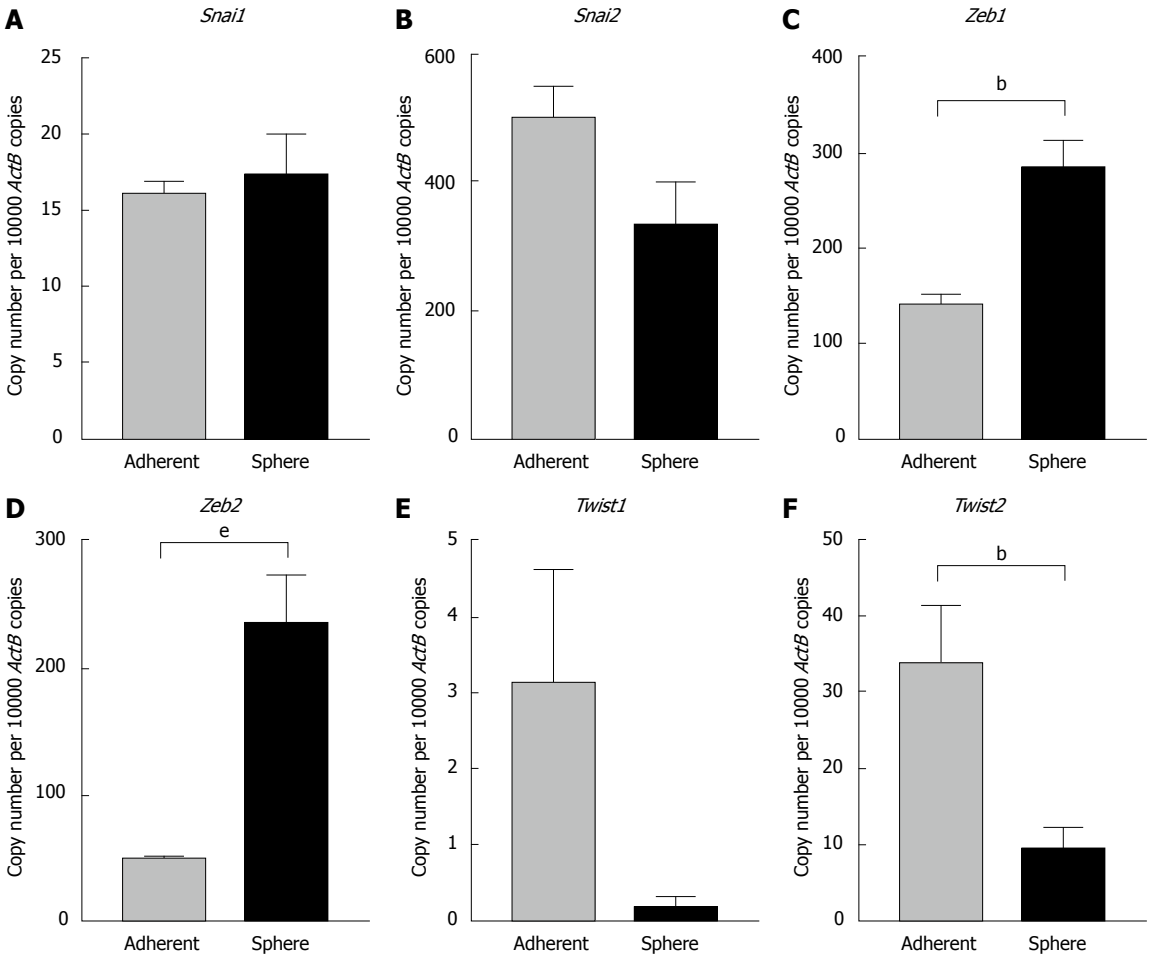

Figure 6 Expression of putative epithelial-to-mesenchymal transition transcription factors in AML12 3-dimensional spheres. qRT-PCR analysis revealed upregulation of A: *Snai1*, C: *Zeb1*, D: *Zeb2* and downregulation of B: *Snai2*, E: *Twist1*, and F: *Twist2* in AML12 3-dimensional spheres compared with AML12 grown as adherent cells. Values are mean ± SEM of three experiments in triplicate ($^{b}P < 0.01$). 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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