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EDITORIAL

Human adult pluripotency: Facts and questions

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Abstract

Cellular reprogramming and induced pluripotent stem cell (IPSC) technology demonstrated the plasticity of adult cell fate, opening a new era of cellular modelling and introducing a versatile therapeutic tool for regenerative medicine. While IPSCs are already involved in clinical trials for various regenerative purposes, critical questions concerning their medium- and long-term genetic and epigenetic stability still need to be answered. Pluripotent stem cells have been described in the last decades in various mammalian and human tissues (such as bone marrow, blood and adipose tissue). We briefly describe the characteristics of human-derived adult stem cells displaying *in vitro* and/or *in vivo* pluripotency while highlighting that the common denominators of their isolation or occurrence within tissue are represented by extreme cellular stress. Spontaneous cellular reprogramming as a survival mechanism favoured by senescence and cellular scarcity could represent an adaptative mechanism. Reprogrammed cells could initiate tissue regeneration or tumour formation dependent on the microenvironment characteristics. Systems biology approaches and lineage tracing within living tissues can be used to clarify the origin of adult pluripotent stem cells and their significance for regeneration and disease.

Key words: Human adult pluripotent stem cells; Induced pluripotent stem cells; Reprogramming; Cellular stress

Core tip: Several types of human adult pluripotent stem cells have been described. Their origin and role remain largely unknown. The elucidation of possible stress-induced pluripotency phenomena could enable regenerative as well as tumour-suppressive therapies.

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INTRODUCTION

During the development of multicellular organisms, cells evolve from the initial undifferentiated, totipotent state of the fertilized egg and early embryo to sequentially restricted states while gradually losing their differentiation potential. The capability of generating more committed progenitors is referred to as the degree of "potency" that defines the "stem" status of a cell. Adult organisms are composed of a large panel of differentiated cell types that accomplish various functions within the body. Among them, a variable amount of tissue-resident stem cells have been documented in various human tissues, accounting for tissue turnover and repair after injury. Tissue specific adult stem cells [such as mesenchymal stem cells (MSCs), neural stem cells, and haematopoietic stem cells] exist in dormant states in adult tissues and are thought to be lineage-restricted, meaning they only give rise to progeny of their tissue of origin. Differentiation implies epigenetic silencing of the so-called pluripotency genes and transcriptional activation of protein-coding genes with cell-type specific functions.

CELLULAR REPROGRAMMING – CHALLENGING THE DOGMA OF LOCKED ADULT DIFFERENTIATION

The ultimate differentiated state associated with loss of cell division, which is known as terminal differentiation, was long considered irreversible. Seminal Nobel-winning research has gradually deconstructed this dogma. Envisaged by Nobel Prize-winner Hans Spemann in 1935, "the fantastical experiment"[1] was performed several decades later. Using the previously established nuclear transfer technology[2], somatic cell nuclei transferred to an enucleated egg cytoplasm were shown to generate a viable adult organism. The experiment confirmed that somatic, fully differentiated adult cells not only retain an intact full genome but can also revert to pluripotent stages under permissive conditions[3]. However, the locked differentiation dogma and definitive rolling down of the epigenetic Waddington landscape[4] was challenged even more dramatically several decades later. In 2006, Shinya Yamanaka used forced expression of several "pluripotency" transcription factors (Oct3/4, Klf4, Sox2 and c-Myc, which was later called the OKSM cocktail) to "reprogram" differentiated somatic cells (mouse fibroblasts) to a cellular status equivalent to embryonic counterparts [embryonic stem cells (ESCs)]. The "reprogrammed" elements, termed induced pluripotent stem cells (IPSCs) were similar to ES cells in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity; the IPSCs were capable of generating various cell types in a teratoma assay and contributed to chimeric animals when injected into mouse blastocysts[5]. The IPSC reprogramming process was further refined to generate cells with potential for germ line transmission[6]. One year later, human fibroblast cells were converted to IPSCs, launching a new era of reprogramming technology with exciting implications in disease modelling and treatment[7]. The initial retroviral-based gene transfer of the OSKM factors was modified to increase reprogramming efficiency and decrease potential tumourigenicity. Non-integrating viruses, stabilized RNAs and proteins, and episomal plasmids are currently used to deliver integration-free reprogramming genes. A large panel of adult somatic or adult stem cells of various species and tissues of origin were shown to be reprogrammable to IPSCs. Some cell types such as neural stem cells required fewer transcription factors for reprogramming[8], while in some cases, small molecules can substitute for the forced expression of one or several OSKM factors[9]. Direct reprogramming of adult differentiated cells to adult cells of different lineages (*e.g.*, fibroblasts to neurons[10]) without conversion to intermediary pluripotent stages was further demonstrated. Using reprogramming methods, adult somatic cells could "go back" to pluripotent stages or directly convert to lineages with distinct developmental origins. Extreme somatic cell plasticity was therefore shown possible under defined *in vitro* conditions. A holistic systems biology approach was applied to existing large "-omic" datasets

from pluripotent cell populations to discover genes important for pluripotency and cell reprogramming[11]. Bioinformatics analysis of several data bases on naïve and primed (pluripotent) ESCs revealed a network of functionally interrelated genes in which the OSKM factors are nodes (Table 1 and Figure 1). Contextual ontology enrichment and quantitative gene expression signatures revealed the mouse pluripotency gene interaction network, the hierarchical importance of genes and pathways, and their significance in pluripotency.

REPROGRAMMING AT WORK

IPSC-based or direct cell reprogramming further advanced to investigating the effect of somatic cell reprogramming *in vivo*.

Short term *in vivo* activation of OKSM factors in transgenic "reprogrammable" mice carrying a tetracycline-inducible OSKM polycistronic cassette crossed with progeria models reduced signs of premature ageing[12]. The same method improved recovery from metabolic disease and muscle injury in older wild-type mice[13]. Cellular epigenetic reprogramming after short-term cyclic *in vivo* activation of OKSM factors (termed partial reprogramming) does not cause tumour formation and probably acts by reverting epigenetic dysregulation associated with older age, offering a platform to study the disease of ageing. In other work, long-term induction of OKSM factors in reprogrammable mice lead to teratoma formation and IPSC induction in a large variety of tissues including haematopoietic lineages. Transcriptomic analysis showed that *in vivo*-produced IPSCs are more similar to ESCs compared to their *in vitro* counterparts; *in vivo*-produced IPSCs are also totipotent as they could generate all embryonic layers and trophectoderm, a property that ESCs are lacking[14]. Intriguingly, *in vivo* forced expression of OSKM factors, a process known to have low efficiency *in vitro*, triggers reprogramming of few cells and induces cellular senescence and apoptosis in many other surrounding cells *in vivo*. Ageing- and tissue injury-associated senescent cell-secreted factors (of which proinflammatory cytokine IL-6 plays a major role) improve the *in vivo* reprogramming process. A similar process might take place under physiological conditions when damage-driven senescent cells promote cell dedifferentiation during tissue repair[15]. *In vivo* direct reprogramming platforms are currently under intense scrutiny and may be the next generation of regenerative approaches for cardiac, neural, liver or pancreatic islet cells. Anti-aging interventions may be a possible outcome of direct somatic cell manipulation[16]. It is worth mentioning that spontaneous reprogramming mechanisms in mammalian organs do occur after injury. Using lineage tracing, several direct conversions were documented in mice. Adult hepatocytes were shown to spontaneously reprogram *in vivo* in biliary epithelial cells after toxic liver injury in a NOTCH-dependent mechanism[17]. Glucagon-producing alpha pancreatic cells converted to beta cells in a mouse model of diphtheria-induced acute selective beta cell loss[18]. Due to obvious ethical constraints, such mechanisms have not yet been documented in humans. Controversial reports about adult pluripotent stem cells in various human tissues prompts reconsideration of their origin and/or causative mechanisms.

ADULT PLURIPOTENT CELL- TYPES AND CONTROVERSIES

Bone marrow-derived pluripotent cells

Starting in the early 2000s, several reports about spontaneously occurring pluripotent cell types emerged. Derived from mice and human bone marrow by negative depletion of CD45 (+)/glycophorin (+) cells, multipotent adult progenitor cells (MAPCs) were reported to undergo triploblastic differentiation under defined conditions *in vitro*. MAPCs did not form teratomas, contributed to chimaera formation when injected into mouse blastocysts, and contributed to cardiac regeneration in severe combined immune-deficient (SCID) mice[19,20]. Marrow-isolated adult multilineage-inducible cells derived from the bone marrow of vertebral bodies under low oxygen conditions were reported to be particularly efficient in differentiating into neural lineages without displaying features of pluripotency[21]. Very small embryonic-like cells (VSELs) were isolated from murine bone marrow by positive selection for the chemokine receptor CXCR4 and were shown to display features of embryonic cells (cell and nuclei size, chromatin characteristics, telomerase activity). The authors hypothesized that such cells with embryonic-like surface markers [stage-specific embryonic antigen (SSEA), OCT-4, and NANOG] could be epiblast-derived pluripotent cell remnants of embryonic developmental stages; these cells could be a less controversial source for regenerative approaches[22]. The existence of VSELs was

Table 1 Pathway population

Cell line-age ident-ificat-ion	Hedg-ehog	P53	WNT target-s	WNT	Cell cycle	VEGF-s	Embr-yonic stem cells	Stem cell signal-ling	Home-obox	Stem cell TF	EMT	Jak-STAT	Stem cells	Termi-nal diff mark-er	Notch-sig	Epige-n chro-m remod fact	Notch target-s	Total-numb-er of pathw-ays
		+	+	+			+			+		+	+					7
			+	+				+			+				+			5
							+	+		+	+	+						5
+			+				+			+			+					5
+			+				+			+								4
+			+									+					+	4
		+			+							+			+			4
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	+							+		+								3
								+	+	+								3
							+			+								2
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					+			+										2
										+						+		2
		+			+													2
										+								1
							+											1
							+											1
3	1	3	5	2	3	0	8	6	2	10	2	5	2	0	2	1	1	56

A list of genes selected from the 1100 upregulated genes in naïve *vs* primed-state embryonic stem cellss. The genes are ranked in ascending order based on the number of scores in different pluripotent-related cell pathways. (+) indicates that the gene is associated with the pluripotency-related pathways listed. Grey highlights the top 10 genes based on their involvement in > 3 pluripotent-related pathways. Three of the 4 Yamanaka factors fall in this list and are marked in bold with*. (Adapted from Mashayekhi *et al*[11]).

challenged a couple of year later as other groups failed to replicate their isolation from bone marrow Remarkably, almost all reports of bone marrow-derived cells that claimed to retain embryonic-like stem cell features were isolated in modified culture conditions (such as low oxygen tension or serum deprivation). Arguments that such cells are early MSC progenitors or culture condition-modified MSCs have not been fully investigated to date[23]. MSCs derived from bone marrow as well as other sources (excluding adipose tissue) were shown to foster a population of SSEA-positive cells with enhanced expansive and clonogenic potential. Arguments that SSEA-positive cells are a culture artefact have not been addressed yet[24]. The existence of adult pluripotent cell populations proved hard to replicate, leading to doubt concerning the accuracy of the reported findings and concept of naturally occurring pluripotency. However, the diversity of reports on enigmatic cells with morphology similar to embryonic counterparts that were isolated under harsh conditions may signal that this is an unelucidated phenomena.

Adipose-derived pluripotent cells

Isolated from adipose-derived stromal vascular fraction, adipose-derived MSCs (ADSCs) were shown to differentiate to non-mesodermal lineages under special culture conditions *in vitro*[25]. Notably, the majority of non-mesenchymal lineage differentiation protocols involve an intermediary step including suspension culture, spheroid formation of intermediary progenitors and sometimes serum deprivation. Undifferentiated or *in vitro* pre-differentiated ADSCs were shown in several reports to contribute to liver, Schwann cell and glial cell regeneration[26]. The advent of IPSCs and the enthusiasm for their potential in generating patient-specific pluripotent cells for research and therapy seemed to throw the controversy of adult pluripotency into oblivion. However, two special cell types continue to capture research interest: multilineage differentiating stress-enduring cells (MUSE) and dedifferentiated fat cells.

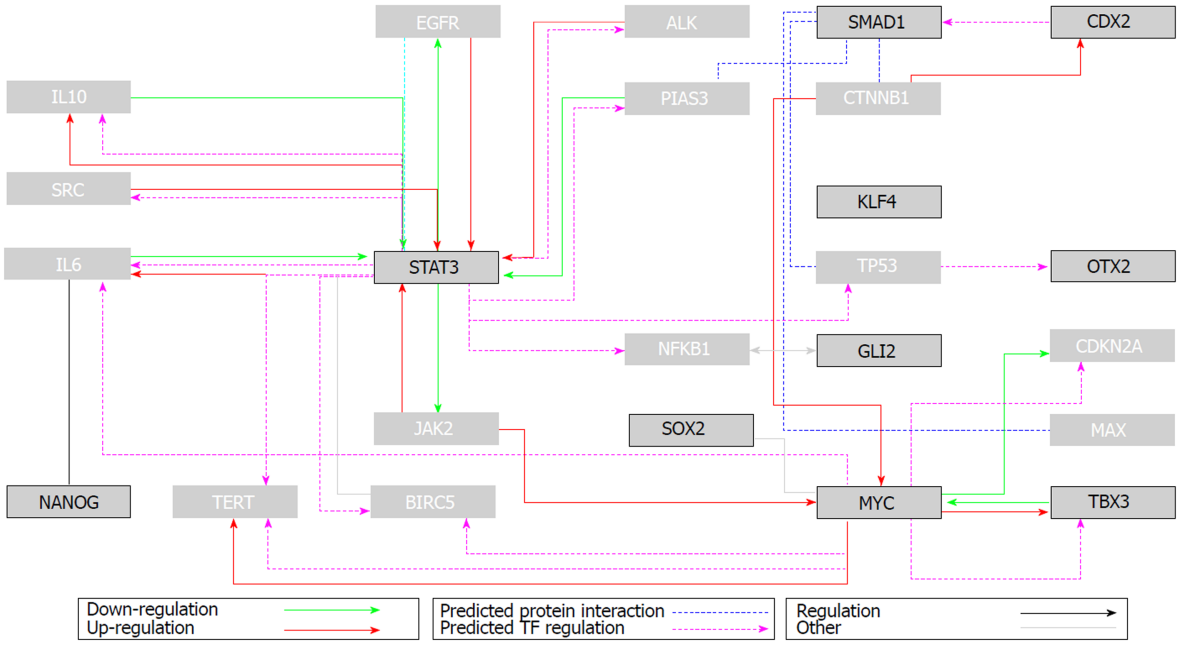

Figure 1 Flux diagram of the top 10 ranked genes related to pluripotency (interaction data obtained from GNCPro, SABiosciences). Interactions: downregulation (green arrow), upregulation (red arrow), predicted transcription factor regulation (magenta arrow), predicted protein interaction (blue line), regulation (black arrow), other types of regulation (grey line). See the electronic version for colour figures. Boxes outlined in black represent the target genes, and light grey boxes their immediate neighbours. Adapted from Mashayekhi *et al*[11].

MUSE cells

MUSE cells were initially identified by applying stressful culture conditions to several cell populations such as MSCs[27,28]; they have been further obtained from adipose tissue by positive immune-separation for the mesenchymal marker CD105 and SSEA-3[29]. MUSE cells are capable of triploblastic differentiation without tumour formation after *in vivo* injection into SCID mice; these were considered safer sources for pluripotent cells than ESCs or IPSCs[30]. With several distinctive properties *in vitro* and *in vivo*, MUSE cells display low telomerase activity and a normal karyotype. MUSE cells form distinctive clusters *in vivo* (the so-called M clusters) that resemble ES or IPSCs behaviours in similar conditions. These cells express pluripotent markers, such as NANOG, Oct3/4, Par-4, and Sox2, and are capable of spontaneous or induced expression of mesodermal, endodermal or ectodermal markers[31]. The low levels of cell proliferation and oncogenesis gene expression might account for their low proliferation and absence of tumourigenic activity, while the expression of gene clusters related to death and survival that are shared with non-mammalian species might represent a highly-conserved mechanism of cell survival during extreme conditions[32]. Several preclinical studies have reported their migratory potential due to expression of chemokines involved in cell homing and their capability to participate in liver, kidney, and neural regeneration in relevant animal models (for review see 30). Muse cells also have immunomodulatory properties in lipopolysaccharide-stimulated macrophages and antigen-challenged T-cell assays through downregulating the secretion of pro-inflammatory cytokines (interferon-γ and tumour necrosis factor-α); this effect is probably acquired by transforming growth factor-β1 expression that decreases the immune-regulatory activity through T-box transcription factors in T cells[33]. Interestingly, MUSE cells have been identified in very low numbers in the blood stream of early stage patients with acute stroke where they probably mobilized from bone marrow; MUSE cells have also been detected *in situ* and in post-mortem bone marrow samples harvested from subjects with severe conditions such as stroke and myocardial infarction[34]. Research to harness the therapeutic potential of such cells for regenerative applications is ongoing; however, their anatomical location in niches has not yet been identified. It is unclear whether induced or naturally occurring stressful conditions are sorting or generating MUSE cells through adaptative and potentially "reprogramming" mechanisms attempting regeneration after major insults.

Adipose tissue was one of the first sources reported for generating MUSE cells and another reportedly pluripotent adult human cell source is dedifferentiated adipose-derived cells (DFATs). Mature adipocytes isolated from adult human adipose tissue

that are subjected to an *in vitro* dedifferentiation strategy (ceiling culture) revert to a more primitive phenotype and gain proliferative and differentiative abilities[35]. Indeed, DFATs were found to have triploblastic differentiation potential *in vitro* and do not generate teratomas when injected in immuno-deficient mice[36]. As opposed to ADSCs that are obtained by enzymatic digestion of adipose tissue and selection of plastic-adherent fibroblastoid elements, DFATs are homogenous populations. DFATS display surface markers for CD13, CD29, CD44, CD90, CD105, CD9, CD166 and CD54, and do not express CD14, CD31, CD34, CD45, CD66b, CD106, CD117, CD133, CD146, CD271, CD309, HLA-DR and alpha-smooth muscle cell actin; a fraction of DFATs also express SSEA-3[37]. Inter-donor and interspecies variability in the makeup of surface antigens has been reported. Combined with a Poly-D, L-lactic-co-glycolic acid scaffold, rat DFAT cells were able to regenerate periodontal tissue[38], opening exciting avenues for oral and maxillofacial tissue regeneration[39,40].

Dedifferentiation as a source of adult pluripotent cells

Mature adipocytes are not the only cells capable of dedifferentiation. Mature chondrocytes isolated from the well organized and highly structured cartilage ECM dedifferentiate while in monolayer culture. When expanded in MSC growth medium with or without fibroblast growth factor (FGF), costal chondrocytes express features of MSCs but retain their chondrogenic potential when injected *in vivo* for cartilage defects[41]. Cartilage progenitor cells with clonogenic and migratory potential reside in osteoarthritic cartilage but not in normal mature cartilage[42]. However, further reports identified surprisingly high levels of the stem cells markers Notch-1, Stro-1 and VCAM-1 in normal cartilage and in a stage- and zone-dependent manner in osteoarthritic (OA) cartilage[43]. Despite their controversial nature, these studies revealed the previously ignored dynamic activity of adult cellular cartilage elements that could be metabolic- and/or mechano-stimulation-dependent[44]. Hypothetically, cells with surface markers of pluripotency in adult cartilage could originate from dedifferentiated chondrocytes induced by metabolic and/or mechanical stress. Disturbances in these parameters might lead to abnormal cell clustering and ECM disorganization that synergizes to produce the progressive cartilage breakdown of OA. The fibrous remodelling of joint surfaces seen in advanced OA stages might represent an abnormal differentiation of such dedifferentiated adult chondrocytes.

Other cell types were shown to successfully dedifferentiate *in vitro* into multipotent or pluripotent progenitors. Adult human thyrocytes regained multipotency, proliferated and differentiated to neurogenic and adipogenic lineages *in vitro*[45]. Terminally differentiated keratinocytes were converted to their progenitor cells under FGF induction[46], while pancreatic islet cells morphed into duct-like progenitor cells under epidermal growth factor exposure[47]. It is noteworthy to mention that these reports involve *in vitro* cell populations. Isolation protocols require breakdown of ECM structures, a process commonly achieved by maintaining cells in monolayer cultures. Intriguingly, reports about *in vivo* formation of DFAT cells after induced local mechanical stress in mice might suggest that this process occurs as a natural adaptative mechanism to local stressful conditions[48]. Dedifferentiation, a common mechanism in plants and a limited number of vertebrates that is used for regeneration, involves switching off genes responsible for cell-specific functions, re-entering the cell cycle and proliferating, and switching on "pluripotency"-related genes. This might be a conserved phenomenon in mammalian organisms including humans. Several factors such as hypoxia, prolonged stress and injury are known to induce dedifferentiated cells after *in vitro* manipulation or *in vivo*. Factors that naturally induce such phenomena *in vivo* and the fate of the regenerative processes they launch need further investigation. Reports about dedifferentiation processes occurring in human malignant tumours, such as liposarcomas dedifferentiating to osteosarcomatous components[49] or soft tissue sarcomas to liposarcomas[50], reflect several rare situations of pathological dedifferentiation processes. Physiological lung myofibroblast dedifferentiation after tissue injury and inflammation accounts for adaptative apoptosis and bronchiolar re-epithelialization. During ageing, impaired dedifferentiation accounts for continued myofibroblast accumulation, excessive matrix deposition and subsequent interstitial lung fibrosis[51].

STAP CONTROVERSY

In early 2014, a paper described a "unique cellular reprogramming phenomenon" of exposing adult differentiated cells to low pH. CD45-positive spleen lymphocytes from 1-week-old C57BL/6 mice carrying an Oct4-gfp transgene and adult cells derived from the brain, skin, muscle, fat, bone marrow, lung and liver that were transiently

exposed to low pH were reported to acquire pluripotency *in vitro*. A portion of such cells, which the authors termed stimulus-triggered acquisition of pluripotency (STAP) stem cells, were shown to express pluripotency markers, differentiate to triploblastic lineages under specified conditions, and contribute to chimaeras and germline transmission when injected into mouse blastocysts. Compared to mouse ESCs, STAP cells displayed limited self-renewal capability in ES-specific media and did not form colonies in dissociated culture[52]. The authors hypostatised that "unknown cellular mechanisms" triggered by sublethal stress unlocked the cells from their differentiated state and allowed re-expression of pluripotency-related genes, reflecting early embryonic stages. Such phenomena do not likely occur *in vivo*-at least not in mammalian organisms-as presumed mechanisms block progression from the initial OCT-4 activation to further reprogramming. Several months later, the paper was retracted due to "errors classified as misconduct" by the institutional investigation committee[53]. The negative impact of the retraction was further combined with news about possible "honour suicide" of one of the senior authors. However, while the "multiple errors" could indeed impact the study reproducibility and the credibility of the reported data, they could not rule out the existence of the STAP phenomenon. Interestingly, a recent paper reported a method of preconditioning adult human umbilical cord blood-derived stem cells to increase survival after transplantation. Exposure to oxidative stress and serum deprivation increased cell resistance *in vitro*, possible pointing to an adaptative mechanism for cell survival[54].

MSCS AND THE "STEM CELL STATE"

The "classical model of hierarchical MSC differentiation depicts a MSC at the top of the potency ladder and subsequent progenies with reduced differentiation potential; this model was challenged by a report showing that murine bone marrow-derived MSCs clonally lost and regained differentiation ability. Fluctuating differentiation potential was even demonstrated at the single-cell level and was closely dependent on culture conditions. Oxygen tension and sparse culture density imposed by clonal expansion altered gene expression and the epigenetic profile that accounts for cell potency. Wnt activation in sparse cultured cells was directly visualized using a green fluorescent protein-tcf/lef reporter, while DNA microarray analysis revealed enrichment of histone methylation in EMT/MET-, MSC-, and Wnt-related genes; this process was found to be oxygen- and tension-dependent[55]. Isolation of cells away from the ECM and exposure to stressful culture conditions might influence mesenchymal cell potency. The authors discussed the potential stress-induced reversibility of cell fate in mammalian mesenchymal cells *in vivo* as a sort of adaptative mechanisms[56].

ADULT PLURIPOTENCY AND AGING

A decline in the reprogramming efficiency of cells derived from older donors in both mice and humans has been reported. However, IPSs with complete set of pluripotency markers as well as differentiation capabilities could be derived from older donors[57,58]. Several epigenetic barriers as well as the increased number of senescent cells, could quantitatively limit the reprograming process in older organisms, process that once initiated remains possible and qualitative. "Aging pathways" (IGF-1 pathway, mTOR,) or "longevity: related ones (AMPK or sirtuins) have been found to influence reprogramming and IPS generation (for review see 57). It is not clear, to date, how these functional and metabolic pathways are involved in potential spontaneous adult cell reprogramming. It is noteworthy to mention that all the above mentioned pathways are highly conserved modalities to sense multidirectional stress (such as energy status, DNA damage, protein damage, and hypoxia) and to orchestrate adaptative cellular responses[59]. Their connection and interference with potential spontaneous induced pluripotency needs to be further studied. Activation of Integrated stress response has been proposed as a mechanism for increased longevity in yeasts[60] and recently was found to facilitate human hematopoetic stem cells survival ans well as to mark leukemia stem cells[61] Cellular stress increasingly appears as a turning point between stemness and malignization in adult as well as aging organisms.

SUMMARY AND SOME QUESTIONS

Reprogramming and the advent of Nobel–awarded IPSC technology has shifted our understanding of cell plasticity. Although previously thought to be "terminally" differentiated, adult cells have been shown to "climb back up" the potency hill to regain multiple differentiation potentials, similar to cells from early developmental stages. The revolutionary technology opened a promising era of cell manipulation for modelling and therapeutic use. IPSCs have gained momentum and are quickly moving towards clinical use for regeneration[57]. Several methods have been proposed to tackle genetic instability forced-reprogrammed cells; however, it their application and utility in designing IPSC-based therapies is not clear[58]. The medium- and long-term fates of therapeutic IPSCs and their progeny after implantation are still unclear, as are the influences on the safety and efficacy of regenerative therapies. The potentially safer alternatives of adult pluripotent stem cells (MUSE, DFAT) have not achieved similar impacts in research interest or clinical translation. Perhaps this is due to their variability, limited reproducibility of production conditions, and poorly explained mechanisms that account for their presence or appearance within adult tissues. It is not clear if adult pluripotent cells already exist in various tissues (such as bone marrow or adipose), or if they are a mere isolation or culture artefact. It remains unknown whether adult pluripotent cells are rare remnants of developmental stages in dormant states within tissues or spontaneously reprogrammed elements. The possibility that severe stress, cell loss or even ageing can induce human adult pluripotent cells both *in vitro* and *in vivo* cannot be ruled out and should warrant further investigation (Figure 2). Can adult "terminally differentiated" cells switch their fate and return to earlier developmental stages in human tissues and during isolation procedures under extreme conditions? If so, what is the threshold of "cellular stability?" Can protocols for steering cellular "destabilization" towards regeneration rather than malignancy be designed with computer modelling? What are the nature and gradient of "stressors" that potentially induce spontaneous reprogramming? What is the role of organism and cellular senescence in promoting or quenching such phenomena? What is the role of immune-mediated inflammation and the senescence-associated inflammatory background in promoting, controlling and halting *in vivo* reprogramming? What potential roles do spontaneously reprogrammed cells have in tissue regeneration and tumour formation? A closer and fundamental investigation of *in vivo* spontaneous-reprogramming phenomena in mammalian cells and humanized animal models could help answer these questions and impact both regenerative medicine and cancer research. Systems biology approaches could be used to discover key switches in biological pathways involved in adult pluripotency and may potentially derive targets for their identification within human tissues.

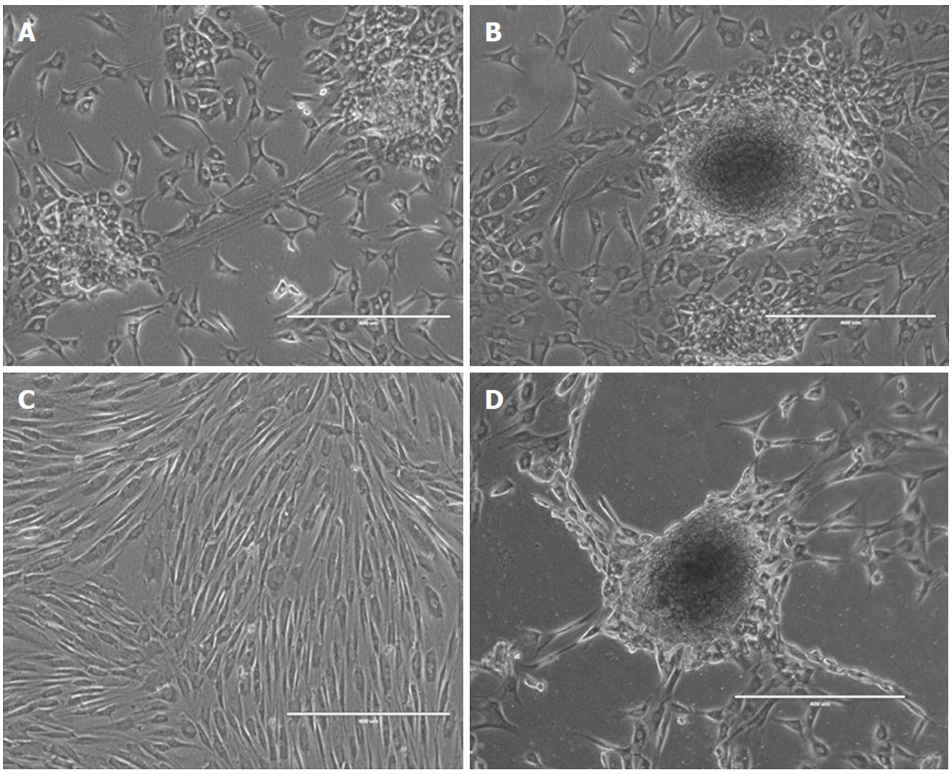

Figure 2 The possibility that severe stress, cell loss or even ageing can induce human adult pluripotent cells both *in vitro* and *in vivo* cannot be ruled out and should warrant further investigation. A: A normal human dermal fibroblast (NHDF) cell line after 48 h of intentional CO_2 absence in the incubator. The cells modified their morphologic characteristics and adopted the culture appearance of pluripotent cells; B: Modified NHDF cells 28 d after stress; C: Non-exposed NHDF cells; D: Modified NHDF cells 73 d after stress.

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REVIEW

Applications of stem cells and bioprinting for potential treatment of diabetes

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Abstract

Currently, there does not exist a strategy that can reduce diabetes and scientists are working towards a cure and innovative approaches by employing stem cell-based therapies. On the other hand, bioprinting technology is a novel therapeutic approach that aims to replace the diseased or lost β-cells, insulin-secreting cells in the pancreas, which can potentially regenerate damaged organs such as the pancreas. Stem cells have the ability to differentiate into various cell lines including insulinproducing cells. However, there are still barriers that hamper the successful differentiation of stem cells into β-cells. In this review, we focus on the potential applications of stem cell research and bioprinting that may be targeted towards replacing the β-cells in the pancreas and may offer approaches towards treatment of diabetes. This review emphasizes on the applicability of employing both stem cells and other cells in 3D bioprinting to generate substitutes for diseased β-cells and recover lost pancreatic functions. The article then proceeds to discuss the overall research done in the field of stem cell-based bioprinting and provides future directions for improving the same for potential applications in diabetic research.

Key words: Bioprinting; Tissue engineering; Pluripotent stem cells; Mesenchymal stem cells; Human embryonic stem; Adult human liver cells; β-cells; Islet cells; Biomaterials; Bioink; Stem cell; Diabetes

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Core tip: The shortage of strategies that can potentially reduce diabetes has prompted scientists to employ stem-cell based therapies that could help generate pancreatic β- cells that can regenerate damaged pancreas. The present review article discusses the potential applications of stem cell research by incorporating 3D bioprinting technology. The article also elaborates the research that has been previously and provides future directions for enhancing the potential applications in diabetic research.

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INTRODUCTION

Diabetes has become a major cause of concern owing to its serious repercussions health and its increasing occurrence at alarming rates. According to the World Health Organization, the number of people with diabetes rose to 422 million and caused 1.6 million deaths in the recent past. Diabetes, a non-communicable disease, is considered as a huge economic burden, for instance in 2010, approximately $376 billion dollars were used to treat and prevent the disease and its complications[1,2]. Over time, diabetes can permanently damage the body organs and is the major cause of kidney failures, heart attacks, strokes, blindness, and lower limb amputations[1,3]. Diabetes is a chronic metabolic disease that can be divided into two main etiopathogenetic categories: Type 1 diabetes mellitus (T1DM), which is the autoimmune destruction of insulin in the pancreas and type 2 diabetes mellitus (T2DM) which occurs when the body uses insulin ineffectively[1].

T1DM, also known as juvenile-onset diabetes, is identified by serological evidence and is most commonly found in infants and children[4]. T1DM is a metabolic disease characterized by the autoimmune destruction of islet beta cells (β-cells) and their secretory functions that result in a deficiency of insulin production[5] (Figure 1A). T1DM involves genetic factors such as human leukocytes, antigen class II genes and environmental factors that initiate autoimmunity[6]. The pathogenesis of T1DM is caused by cellular and humoral immune pathways where $CD8^+$T lymphocytes kill β-cells[1]. T1DM patients do not produce insulin and exogenous insulin administration is required to mimic insulin release to control glucose levels during mealtimes. Patients with T1DM have been treated with immunosuppressant agents in the past, but this type of treatment does not maintain the function of β-cells rendering insulin replacement therapy as the only treatment effective for the restoration of metabolic disturbances in T1DM patients. The treatment for T1DM requires administering a long acting insulin dosage (once or twice a day with each meal)[7]. Furthermore, treatment for T1DM is based on a rigorous monitoring of blood glucose levels and intravenous insulin injections. The management of T1DM requires significant patient compliance, which is associated with an increased risk of hypoglycemia.

T2DM or adult-onset diabetes is a more prevalent category caused by a combination of insulin resistance and inadequate insulin secretory responses and functions[1,4] (Figure 1B). T2DM is asymptomatic as its progression causes hyperglycemia, which triggers pathological and functional changes in target tissues. Patients with T2DM and T1DM are at risk of developing micro- and macro-vascular complications[8-13]. These are associated with atherosclerotic disease affecting arteries that supply blood to the heart and increase the risk of cardiovascular disease in which death from myocardial infarction and strokes is the leading cause of mortality in T1DM and T2DM patients[2,14]. Patients with T2DM are subjected to a non-insulin-based therapy and in some patients with T2DM; the insulin requirements are similar to those with T1DM necessitating daily injections for long-acting insulin during mealtimes[15].

Researchers have intensely studied diabetes and have decades of experience investigating means to replace β-cells of the pancreas that are destroyed by the immune system. Current procedures involve allotransplantation, which requires passing a catheter through the liver, involving high risks of bleeding and blood clots, and is categorized under extremely invasive surgeries[16]. A very common treatment for diabetes is the transplantation of the pancreas, but an extreme shortage of donors still exists[9]. According to United Network for Organ Sharing, a person is added to the

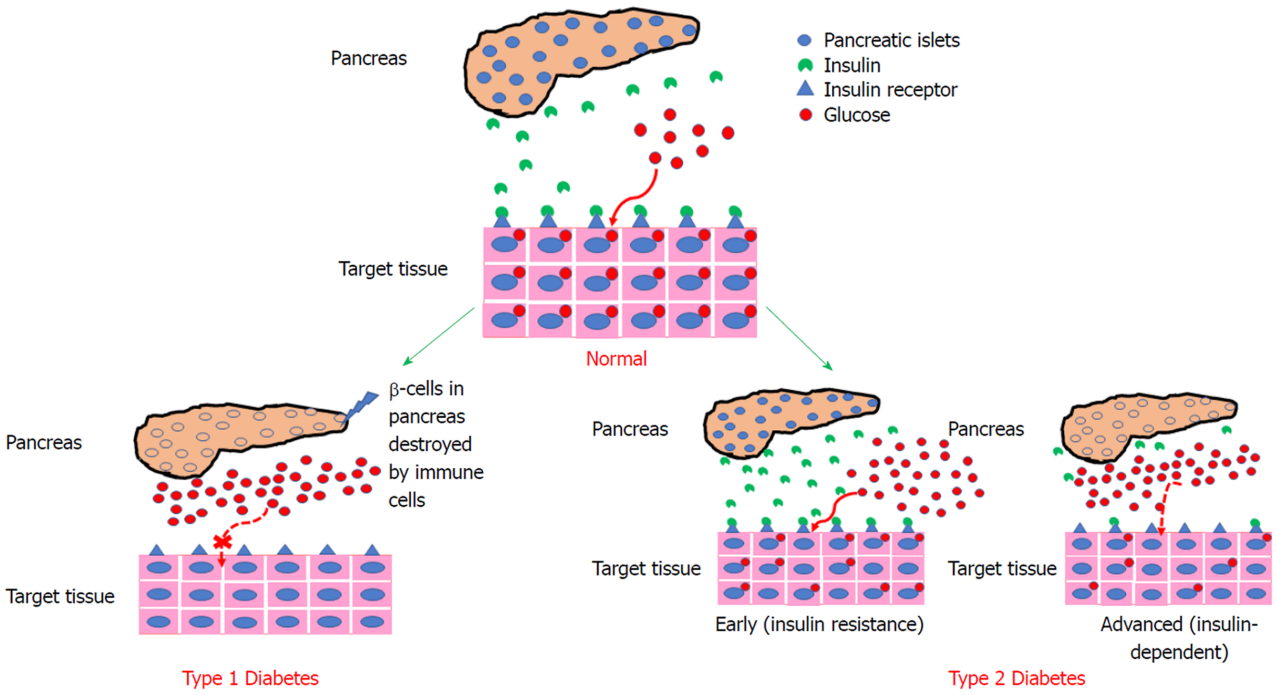

Figure 1 Schematic representation differentiating between normal and diabetic (Type 1 and Type 2) pancreas.

national transplant waiting list every ten minutes. Moreover, the other hurdles related to pancreas and islet transplantation are associated with alloimmune responses[9,16].

Current treatment strategies have not been able to successfully maintain or replace the function of β-cells, thereby seeking alternative therapies such as regenerative medicine using stem cells, in combination with bioprinting technologies to cure and diminish the health challenges of diabetes. The efficacy of combining stem cells and additive manufacturing in the field of regenerative medicine has been established in prior studies and has prompted further research for scientists, worldwide. This review discusses stem cell-based therapies and the applications of bioprinting in regenerative medicine, which can be directed towards strategizing potential treatments for regenerating the pancreas affected during diabetes, either in part or as a whole.

STEM CELL-BASED THERAPIES

The main goal of diabetes therapy is to attain normoglycemia through the replacement of the diseased or lost cells of the pancreas with new cells. Scientists have attained success in producing insulin-secreting cells from different types of cells. This section focuses on the current types of stem cell research to treat diabetes and past research relating to novel applications of stem cell therapies for diabetes.

Stem cell-based therapy

Induced pluripotent stem cells: Ever since Takahashi *et al*[17] demonstrated that induced pluripotent stem cells (iPSCs) could be generated from differentiated somatic cells through the reprogramming of adult and embryonic mouse fibroblasts by transfecting the cells with plasmids, they have opened up a possibility for replacement in cell-based therapy. iPSCs are also favored for their capacity to self-renew infinitely and their potential for differentiation into a wide variety of cell types[18]. The maintenance of undifferentiated iPSCs as cell lines holds great promise for modeling diseases and to generate personalized stem cells for cell therapies[19]. According to studies done by Alipio *et al*[20], hyperglycemia in diabetic mice was found to be controlled by mouse skin fibroblast-derived iPSCs that differentiated into β-like cells, which were morphologically identical to normal, endogenous cells that secreted insulin. Mature pancreatic cells that had the ability to secrete insulin and C-peptide were generated by the differentiation of human embryonic stem cells (ESCs) and iPSCs[21]. Patients suffering from T1DM and T2DM diabetes were employed as sources to produce iPSCs[22]. *In vitro* production of insulin-secreting cells was also achieved by the directed differentiation of iPSCs using small molecules and growth factors in the

culture[23]. The primary advantages of employing iPSCs are that they do not present ethical concerns and only pose a low risk of teratoma formations[24]. However, the reprogramming of somatic cells into iPSCs achieved with the aid of viral transfection of transcription factors requires the use of genomes[25]. These genomes are harmful as they can trigger mutations and hamper the normal function of iPSCs and their ability to differentiate, in addition to causing the formation of tumors[25].

Mesenchymal stem cells: The method for isolating mesenchymal stem cells (MSCs) from the rat bone marrow was first described by Friedenstein as explained in previous studies[26]. Although the bone marrow is the richest source of MSCs[27-29], they have also been successfully isolated from adipose tissues[30,31], fetal liver[32], umbilical cord and its blood[33,34], fibroblasts[35], endometrium[36], placenta[37], trabecular and compact bone[38]. MSCs have been found to be able to differentiate into mesodermal, endodermal and ectodermal cells under suitable culture conditions[39]. MSCs are suitable for the regeneration of tissues, as they do not result in teratoma formation[39]. Other advantages of using MSCs for stem cell-based therapy include the ease of isolation, expansion to large quantities and their multipotential differentiation capacity[40]. In addition, their ability to circumvent immune recognition and inhibit immune responses also makes them ideal candidates for immunomodulatory cell therapy in immune-mediated diseases[41].

According to studies performed by Xu *et al*[42], the direct injection of MSCs into the pancreas had helped alleviate diabetes symptoms by improving the metabolic control in animal models, counteracting autoimmunity, enhancing islet engraftment and survival, besides serving as a source of growth factors and cytokines. Direct injection of MSCs has not only been found to be effective in improving the functions of the pancreas but also healed related symptoms like diabetic foot and neuropathy[43]. The main limitation posed by MSCs is their potential to differentiate into unwanted mesenchymal lineages, which can be detrimental to their therapeutic applications[44]. The possibility of malignant transformations and cytogenetic aberrations of MSCs may also considered drawbacks[44]. Results of some MSCs clinical trials in T1DM are shown in Table 1[45-51].

Human embryonic stem cells (hESCs): hESCs are characterized by properties such as pluripotency of gene expression, self-renewal ability, and high proliferative capacity[52,53] thereby making them a valuable treatment option in all types of medicine. Numerous *in vivo* and *in vitro* differentiation strategies have been adopted for the production of functional pancreatic islets. Generally, hESCs are initially harvested from the inner cell mass of the blastula post fertilization when the cells are still capable of differentiation into all types of germ layers and there is a high level of telomerase activity[52]. This is followed by the differentiation of the hESCs into definitive endoderm, which further undergo differentiation into functional β-cells, through a chain of endodermal intermediates[54,55]. These techniques cause the hESCs to be exposed to specific transcription factors that can facilitate coordinated activation and inhibit intracellular signaling pathways. Although cell signaling and epigenetic factors involved in the differentiation process remain to be studied and understood, the detection of markers such as pancreatic and duodenal homeobox gene 1 (PDx1), insulin gene enhancer protein (Isl-1), and Forkhead box protein A2 validate the endodermic differentiation into endocrine and exocrine pancreatic β-cells[56,57].

Non-stem cell-based therapy

Adult human liver cells: The liver has been extensively studied as a potential source for pancreatic β-cells that can help cure diabetes. It has an added advantage over other organs as it has been derived from the endoderm along with the pancreas[58,59]. A comprehensive developmental shift of adult human liver cells into insulin-producing cells was induced with the help of PDx1 and other soluble factors[59]. Studies conducted by Yang *et al*[60] provide evidence that purified adult rat hepatic oval ''stem'' cells transdifferentiate into pancreatic endocrine hormone-producing cells when subjected to culture in a high-glucose environment. These differentiated cells then self-assemble forming three-dimensional islet cell-like clusters that express pancreatic islet cell differentiation-related transcripts which can be validated by reverse transcription–PCR/nested PC and islet-specific hormones detectable by immunohistochemistry[60]. Hepatic oval cell activation through hepatic trans-differentiation and pancreatic islet regeneration was also successfully reversed for streptozotocin-induced diabetes[61]. Although these methods differed in terms of their approaches, they were successful in ameliorating hyperglycemia in the mouse models. This further led to a search for alternate pancreatic sources of insulin as can be seen from the studies conducted by Zalzman *et al*[62], which demonstrated the reversal of hyperglycemia in mice by employing human expandable insulin-

Table 1 Results of some mesenchymal stem cells clinical trials in diabetes mellitus type 1[45]

Types	Routes of transplantation	Outcome
Human MSCs	Intravenously introduced to Non-obese diabetic/Severe combined immunodeficiency mice with total body irradiation or local abdominal or leg irradiation	Safe and efficient for the long-term treatment of severe complication after radiotherapy[46]
Umbilical cord derived MSCs	Injected directly into the pancreas	Improvement of metabolic control. Enhancement of islet engraftment and survival[42]
Bone marrow-derived MSC	Differentiated *in vivo* into functioning β-cells	Normalization of chronic hyperglycemia in a diabetic rat[47]
Human placenta derived MSCs	Differentiated into islet-like cell clusters and transplanted into streptozocin-induced diabetic mice	Restoration of normoglycemia in diabetic mice[48]
Human umbilical cord blood derived MSCs	Differentiated into IPC through intravenous administration	Improvement in glycemic profiles, histological improvement of insulates[49]
Wharton's jelly and amniotic membrane derived MSCs	(1) Differentiated into IPC and transplanted into the liver; (2) Infected with *PDX1* gene and differentiated to IPC; and (3) Differentiated into IPC and transplanted into the liver of STZ-induced diabetic rats	Expression of insulin Secretion of C-peptide; expression of pancreas-specific genes[49]; correspondence to high concentrations of glucose[50]; reduction of blood glucose levels after 4 wk of transplantation[51]

MSCs: Mesenchymal stem cells; IPC: Insulin-producing cells.

producing cells that were generated by the differentiation of fetal liver progenitor cells .

β-cells: The pancreas is the first choice for harvesting potential stem cells for the treatment of diabetes[63]. Bonner-Weir *et al*[63] demonstrated through their experiments that the availability of small amounts of pancreatic tissue could help to restore the maximum pancreatic β-cell mass. This has been attributed to the replication and de-differentiation of differentiated β-cells of the pancreatic ducts, which in turn triggers the production of more β-cells. Further studies conducted showed that these ductal cell populations could be cultivated and directed into forming cell-clusters secreting insulin[63,64]. A clonal population of adult pancreatic precursor cells, that had the ability to produce both insulin and C-peptide, were generated from ductal cells by Seaberg *et al*[65]. Although there were debates in the past about the existence of pancreatic adult stem cells despite their progress and potential, strong evidence indicating that the pancreatic ducts of mice contained multipotent progenitor stem cells, which could generate new β-cells, was given by Xu *et al*[66]. However, more research needs to be done for the promotion of β-cell formation in diabetic patients by finding and activating pancreatic stem cells. This necessitates the development of better experimental strategies to come up with suitable methods to overcome the issues of isolation and ex-vivo expansion of these stem cells for transplantation.

Islet cells: The pancreatic islets, also termed, as the islets of Langerhans, constitute regions of the pancreas that contain the hormone-producing cells (endocrine cells) and were first described by Paul Langerhans in 1869, a German pathological anatomist[67]. The relation between the pancreas and diabetes was established much later by Minkowski and von Mering[68]. The islets of Langerhans were first isolated from the pancreas of a guinea pig by Moskalewski *et al*[69] by employing an enzymatic digestion technique. Studies conducted by Bottazzo *et al*[70] indicated the possibility that islet cell transplantation would be a very suitable option for people who were suffering from T1DM poorly controlled with insulin. The challenges of transplanting islet cells include finding compatible donors, ensuring the survival of the new islets and side effects induced by medications administered to prevent immune rejection[71]. Azarpira *et al*[72] successfully isolated islet cells from cadaveric donors which were then administered *via* injections into the recipient's portal vein. The study showed that there was a reduction in the initial β-cell mass attributed to instant blood-mediated inflammatory reactions, immune responses resulting from the transplantation of the islet cells and diabetogenic effects triggered by the immunosuppressive medications[72]. This necessitated the need for repeated episodes of cell transplantation to ensure significant outcomes[73]. According to studies conducted by Bennet *et al*[74], it was established that the exposure of isolated islets to ABO-compatible blood resulted in an immediate thrombotic reaction and hence required multiple transplants to reduce the insulin shots. There was also the possibility of the impairment in insulin production of the transplanted islets due to

their entrapment by blood clots, which could shut them off from oxygen and attract immunocytes[75]. This motivated scientists to seek alternative cell sources such as pluripotent and multipotent stem cells, to generate pancreatic cells and aid in diabetes therapy by replacing the diseased or lost pancreatic cells[76].

3D BIOPRINTING

Bioprinting techniques had emerged in 1988, as demonstrated by Klebe[10] using cytoscribing technology, a method that requires mispositioning of the cells to construct synthetic tissues using a Hewlett Packard inkjet printer. 3D bioprinting is a revolutionary field that is utilized in biomedical engineering and sciences. The difference between 3D printing and 3D bioprinting is that bioprinting technologies utilize living cells, which are printed layer by layer to form a 3D structures[11,12] with the ultimate goal to regenerate the diseased or damaged tissue and reduce organ shortage[2]. Currently in the United States, there is a great need for an alternative to organ transplants, due to the limited availability of organ donors[77]. A potential solution for this problem is tissue engineering by developing organs that can be built with the patients' genetics to eliminate the chances of rejection, relieve suffering, and save lives[78]. The purpose of tissue engineering and state-of-the-art 3D printing is to develop a degradable scaffold, that will allow cells to proliferate and regenerate through pores to replace the damaged organ or tissue. These characteristics provide the cells with viability and functionality, in addition to the ability to attach and mimic the native organ environment[13].

3D printing tissue engineering and regenerative medicine holds great promise for building and assembling viable and functional tissues and organs. 3D printing involves a combination of scaffold and biomolecules that sustain the cells, to improve or regenerate specific tissue or the whole organ[13]. Researches had encountered challenges while trying to develop the accurate scaffold materials for manual cell seeding[79]. Difficulties in seeding the cells manually limit the cells' precise placement and ability to proliferate inside the scaffold[79]. Despite the great advantages of biofabrication of scaffolds, another limitation is that cells need to grow in high density to develop the thickness of the organ or tissue, which is difficult to achieve because the cells only attach to the surface and do not penetrate the entire scaffold[79]. Furthermore, the difficulty and need to achieve vascularization and anastomosis is critical. These challenges have led to the development of optimization of bioprinting technologies and cell seeding protocol where scientists encapsulate large numbers of cells to achieve density and promote oxygenation, vascularization and the desired pattern through the scaffold[80,81].

3D bioprinting technologies involve the design of unconventional scaffolds where the design is inspired by the patient's own anatomy for developing a correct shape for the tissue construct. Bioprinting technology can develop a porous construct to allow media and nutrients to reach the cells. Bioprinting technologies are based on three major steps for the design of tissue regeneration. To develop a medical image of the desired area of the body, a blueprint is created using a software system, which is followed by toolpath planning and finally 3D bioprinting, which is divided, into three major categories depending on the technique employed to print (Figure 2)[13,79,82].

The first category is extrusion-based bioprinting that uses a combination of automated robotic and fluid allotting system of pneumatic, mechanical force or solenoid micro-extrusion to continuously extrude bioink on the biopaper[13,79]. The second category is inkjet-based

bioprinting, in which small droplets of cells are ejected to fabricated tissues[83]. This method involves electro-hydrodynamic jetting, acoustic droplet ejection, thermal, piezoelectric, or electrostatic energy for printing[79]. The third category is laser-based bioprinting; which incolves cell-transfer and a photo-polymerization process using digital light to crosslink the bioink (Figure 2)[13,79].

The process of bioprinting involves two components, namely the bioink and the biopaper. The bioink is a biomaterial in which live cells are embedded to print on the biopaper to mimic the extracellular matrix of the desired tissue. The biopaper is another important component of 3D bioprinting because it serves as the substrate on which cells (bioink) are deposited in an organized pattern[13]. Currently, hydrogels are popularly employed as bioinks as they facilitate effective oxygen, nutrient and metabolite transportation, besides providing great permeability to water[13,79,84].

However, synthetic bioinks struggle to achieve high printability and biocompatibility, thereby strengthening the need for developing naturally derived bioinks. A novel furfuryl-gelatin based bioink was developed and found to exhibit a highly porous networked structure, and co-culture feasibility when C2C12 myoblasts

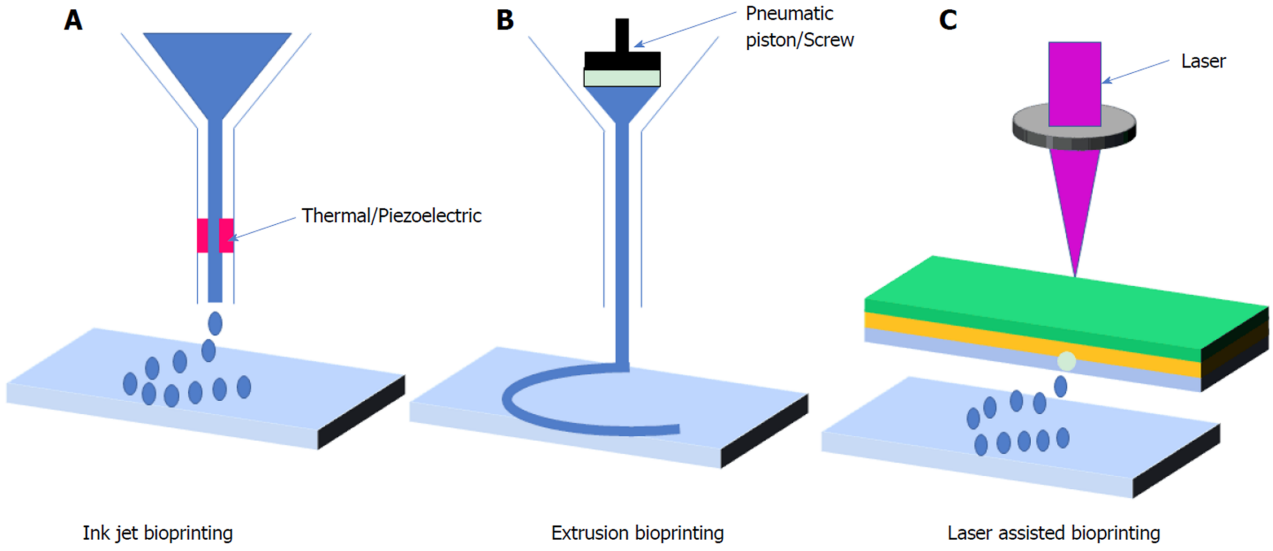

Figure 2 Classification of bioprinting techniques. Three major classifications of bioprinting modalities are A: Inkjet-based printing, which air-pressure pulses that force droplets from nozzle by heating up the printhead; B: Exzrusion-based printing,using pneumatic or mechanical dispensing systems for extruding continuous beads of materials and/or cells; C: Laser-based bioprinting that uses lasers focused on an absorbing substrate for generating pressure that compels the bioink to be extruded onto a collector substrate.

and STO fibroblasts were printed in a double-layered structure[85]. These structures, cross-linked by exposure to visible light, have been successful in preserving the viability of both cells types, showing that this bioink can be used for tissue engineering applications for developing complex tissues to help study cellular communication in a disease or normal models[85]. Comparison of cell viabilities for ink jet based-, extrusion based- and laser assisted bioprinting is shown in Table 2[86-90].

Other properties of the bioink, such as transfer of thermal energy into kinetic energy and high viscosity, rapid gelation mechanism by enzymatic, physical, or chemical crosslinking processes are important for consideration to develop the ideal scaffolds[91].

APPLICATIONS OF BIOPRINTING

3D bioprinting has the ability to write living cells in a stackable layer-by-layer organizational pattern using biomaterials to engineer a specific construct for the use of tissue regeneration, surgery procedures, drug and medical studies to treat disease and health-related complications[84,92,93]. This computer-assisted technology is a powerful tool that has obtained attention worldwide[79] and 3D bioprinting modalities are driven by endless possibilities of innovative use in regenerative medicine and tissue engineering. This technology offers the advantage of placing cells in a precise location and specific fashion to create a cellular models[84,92,93].

Tissue engineering and regenerative medicine

Current translational benefits in 3D bioprinting are in tissue engineering and regenerative medicine *i.e.*, bone tissue engineering for the development of the specific tissue construct by recreating the unique patients' anatomy[79,94]. Another such benefit of 3D bioprinting is the ability to develop a cardiac patch with the ability to synchronously beat, which has great promise in regenerating a specific area of the heart[95]. Anil Kumar *et al*[85] developed a novel furfuryl-gelatin based hydrogel that was bioprinted into cell-laden rectangular constructs and may potentially be implanted on post infarcted hearts. Cartilage tissue has been successfully bioprinted, to solve cartilage defect repair[65]. Furthermore, the progress made in creating an organ-in-a-chip helps to simulate the mechanisms and functions of a specific body area[92]. This approach also provides the opportunity to perform drug screening studies for diseases[96]. However, the difficulty in incorporating vascularization simultaneously with the 3D bioprinting of tissues, gives rise to challenges in the fabrication of bone tissues, to treat major defects or bone loss[97]. The use of hydrogels for bone tissue in bioprinting approaches makes it difficult to implant into a load-bearing site in the patient's body[98]. Thus, the hydrogels need to be mechanically robust and possess the characteristics to support large-scale regeneration of bone

Table 2 Comparison of cell viabilities for ink jet based-, extrusion based- and laser assisted bioprinting[90]

3D bioprinting technique	Cell viability
Ink jet based bioprinting	80%-95 %[86,87]
Extrusion based bioprinting	89.46% ± 2.51%[88]
Laser assisted bioprinting	< 85%[89]

tissue *in vivo*[98].

3D bioprinting for bone tissue reconstruction presents major challenges related to vascularization. Considerable progress has been made in skin bioprinting, but improvement in scar-less tissue formation need to be implemented[79]. Another challenge that needs to be overcome involves *in vivo* studies for bioprinted blood vessel and the organ fabrication. Moreover, the availability of technologies capable of bioprinting vascular networks in high density and generate organ constructs integrating different tissues together, are also needed[79]. For this, co-culture of different cell types for the development and reconstitution of the functionality of a whole organ is necessary. Despite the progress in 3D bioprinting technologies translated from bench to bedside, the aforementioned applications still have limitations and challenges that need to be overcome, especially in the fabrication of functional tissues with long-term viability[99]. For instance, the heart, pancreas, and liver are the organs that are the most difficult to fabricate due to the need for metabolic functions and vascularization[79]. Metabolically highly active organs are a great challenge to reconstruct because their complexity requires molecular networks from arteries, veins and cell communication of different cell types in order to mimic the identical long-term functionality[99].

Currently, bioprinted living constructs have been acutely investigated and transplanted *in vivo* (animal models)[100]. Animal studies provided the opportunity and insights into evaluation of engraftment of the implant with the host anastomosis, vascularization, and regeneration of functionality[101]. 3D-printed metallic, plastics, and ceramics have been developed as successful constructs for bone tissue replacement and these constructs have been transplanted into humans[97]. Bioprinting is a powerful tool for medical procedures, especially for a near future with possible in situ bioprinting[79]. In situ bioprinting is an attractive application for 3D bioprinting that has provided a major advantage in regenerative medicine over traditional procedures. Recently, the use of in situ bioprinting was applied in skin regeneration for large wounds on pig models[102] and skull defects in rodents[101,103]. The advancement of in situ bioprinting can be applied in the regeneration of a variety of tissues and organs such as plastic surgery, maxillo- and craniofacial reconstruction[103].

Screening and drug toxicity testing

Another benefit in using bioprinting technologies is the application of bioprinted tissue and organ models for potential pharmaceutics use and for screening and drug toxicity testing[96]. This application relieves the time consumption and cost related to drug discovery, which entails financial investment and human resources. In addition, 3D bioprinted tissues have the ability to bioprint in microarrays and develop *in vitro* 3D-pinted models that mimic the native human tissue[79,96]. This approach provides the opportunity to use a 3D-assay system that may contribute to a possible solution to lower the cost and financial investment in pharmaceutics. Bioprinting has also offered other great advantages for testing toxicity; for instance, the development of liver-on-a-chip for testing hepatic toxicity of acetaminophen[104] and the test of antitumor drugs for breast cancer[105].

Future concerns

Regenerative medicine is a rapidly expanding area of research that deals with repairing or replacing damaged tissues and organs[106]. Tissue engineering may one day put an end to allogenic organ transplantation and the need for immunosuppression. Stem cells are a cornerstone to this process, as they possess the ability to differentiate into nearly any cell type[107]. Combining the abovementioned research fields with 3D bioprinting will allow for *in vitro* tissue creation. Bioprinting uses the 3D additive manufacturing process while utilizing biomaterials, growth factors, or different cell types as the printing medium[108].

Computed tomography or magnetic resonance imaging scans can be used to create a digital blueprint of the desired organ[109,110]. This computer created file is then converted into thin slices that can be layered on top of one another. When the 3D

printing process is done the tissue still needs to undergo a maturation process before it can be implanted. Over time the tissue will start to develop its own extracellular matrix and any temporary scaffolding is degraded[110].

One of the largest challenges in 3D printing human tissue and organs is to implement and promote vascularization[111]. Researches have tried to overcome this obstacle by printing sacrificial mediums embedded in endothelial cells, which can mature into blood vessels as the original medium slowly degrades over time[112]. However, these constructs are extremely fragile and require mechanical and chemical stimulation to undergo maturation and capable of implantation into the body[113]. Once in the body, the new tissue must generate its own extracellular matrix to be fully incorporated. Trauma and tumor growth can lead to substantial amounts of bone loss[114]. Traditional bone grafts are limited < 5 cm in size and often fail due to residual stress[115]. Gao *et al*[87] used an inkjet printer to print peptides and PEG with simultaneous photo-polymerization using bone marrow mesenchymal stem cells, which showed significantly enhanced osteogenic differentiation.

Liver transplantation is the only cure for liver failure. However, there are more people waiting for livers than there are donors, leading to many deaths while waiting for a transplant[116]. Faulkner-Jones *et al*[117] differentiated iPSCs into hepatocytes after bioprinting showed that stem cells maintain their pluripotency during the printing process. Ahn *et al*[118] printed a multilayer porous mesh structure made with alginate and ADSCs, which they successfully differentiated into a hepatogenic lineage expressing liver-specific genes.

THERAPEUTIC APPLICATIONS OF STEM CELLS AND BIOPRINTING TOWARDS DIABETES

Around 15 different types of tissues have been studied in bioprinting technology but there are other tissues types that are part of the human body, which are unexplored and need more investigation[79]. In addition, the innovation of bionic organs or new types of organs is a possible direction for the future in bioprinting to solve organ shortage and alleviate patients' suffering[119]. Bioprinting research involves multiple cell types patterned to mimic the complex anatomy of the human body and the understanding for an optimal protocol for culture conditions with multiple cell types; these optimizations should include the correct medium and nutrients to promote growth and viability of multiple cell types[79,81].

For T2DM

An example of an application of bioprinting with cells is a pancreatic model bioprinted with pancreatic islets that was implanted into a diabetic murine model leading to regulated insulin secretion. However, the size of the mouse model of study was significantly different, about 100000 times smaller than a human model[79,100,120]. Hence, the 3D bioprinted models of study need to have relevant dimensions for clinical use *i.e.*, the simulation of human size, a larger animal model needs to be used that can possibly represent human physiology[121].

A recent study had reported translational benefits of adult and embryonic stem cell in which stem cells can be used to produce insulin-like secreting cells known as β-cells[76]. The translational benefits provided evidence towards the existence of new β-cells generated by the replication of pre-existing β-cells from the adult pancreas or partial removal of the pancreas[76]. Cells used to reconstruct and regenerate the pancreas after implantation must be pathogen free. Ideally, the cells that will differentiate into β-cells should not only be able to reconstitute the function of the pancreas but also maintain long-term and normal activity[122]. It has been shown that mature exocrine cells of the pancreas can be reprogrammed to become β-like-cells *in vivo* with a combination of 3 transcription factors[123]. Another challenge that needs to be addressed is that the differentiated β-cells persist as individual cells or small clusters and do not reorganize into islets before clinical therapy is induced[122]. The viruses that are used to reprogram factors needed for induction of differentiation should be replaced with safer reagents to produce β-cells[124].

Although 3D bioprinting has been successfully applied to fabricate tissues such as blood vessels[125,126], skin[127,128], bone and cartilage[93,122,125-130] and liver[13], the bioprinting of pancreatic islet tissues to treat diabetes remains to be explored. However, other techniques such as stereolithography have shown promise, in this regard. According to work done by Gallego-Perez *et al*[131], microwell arrays were created with stereolithography and electrospinning, and structurally interfaced with a porous sheet of micro/nano-scale polyblend fibers. These arrays served as a platform for the anchoring and subsequent assemblage of human pancreatic ductal epithelial cells into

insulin-expressing 3D clusters occurred[131]. Given that cluster size and uniformity are known to influence islet cell behavior, the ability to effectively control these parameters could find applications in the development of anti-diabetic therapies[131]. Immunoreactivity for insulin, C-peptide and glucagon was detected on both the platform and control surfaces; however, intracellular levels of C-peptide/cell were approximately 60% higher on the platform[131]. Alginate-based porous scaffolds as extra-hepatic islet delivery systems were successfully developed through 3D plotting by Marchioli *et al*[99]. INS1E β-cells, human and mouse islets were successfully embedded in these 3D-plotted constructs without affecting their morphology and viability while preventing their aggregation[99]. Studies such as these show that there is a definite possibility of treating diabetes by incorporating 3D printing technology, but rigorous research is in order before that can be achieved.

Investigations led by Dor *et al*[132] provided conclusive evidence that terminally differentiated β-cells could retain a significant proliferative capacity *in vivo* and could be used as a major source for new β-cells during adult life and following pancreatectomy in mice.

A scalable differentiation protocol to generate millions of glucose-responsive β-cells from hPSC *in vitro* was reported by Pagliuca *et al*[54] as the, insulin-producing cells that were previously generated from human pluripotent stem cells (hPSC) were found to lack many functional characteristics exhibited by bona fide β-cells.

Ozbolat *et al*[14] proposed the concept of miniature organs, that could potentially be fabricated on a smaller scale in comparison to their natural counterparts and closely mimic the most vital function of the associated organ, such as a pancreatic organ. This organ could be placed in a less immune-responsive site in the body to effectively produce and secrete insulin in the desired quantities into the bloodstream to regulate glucose levels to normoglycemia in the human body[14].

Chen *et al*[133] investigated the possibility of differentiating rat marrow MSCs *in vitro* into functional islet-like cells and to confirm their diabetes therapeutic potential. Insulin mRNA and protein expressions were observed in the resulting typical islet-like clustered cells[133]. The insulin excreted from the differentiated cells was found to be much higher than the undifferentiated MSCs[133]. The injected differentiated MSCs were also found to downregulate glucose levels in diabetic rats when diabetic rat models were made to test the *in vivo* function of the differentiated MSCs[133].

Jiang *et al*[56] established a novel serum-free protocol to generate insulin-producing islet-like clusters (ILCs) from hESCs grown under feeder-free conditions. The hESCs were treated with sodium butyrate and activin A to generate definitive endoderm[56]. The endoderm population was then converted into cellular aggregates which were further differentiated into Pdx1-expressing pancreatic endoderm in the presence of epidermal and basic fibroblast growth factors[56]. The aggregates were finally allowed to mature and the temporal pattern of pancreas-specific expression in the hESC-derived ILCs showed considerable resemblance to *in vivo* pancreas development, and the final population contained representatives of the ductal, exocrine, and endocrine pancreas[56].

Ferrell *et al*[134] successfully developed a technique that could enable the active patterning of individual cells and groups of cells in a polymer-based microdevice using vacuum-assisted cell seeding. Polymer microwells with various geometries on top of commercially available porous membranes were moulded by employing soft lithography[134]. This method was used to determine the number of cells in a microwell for given cell seeding density and microwell geometry and tested successfully with pancreatic ductal epithelial-like cells indicating potential applications in tissue engineering[134].

Patients with diabetes mellitus are at a greater risk of developing heart failure such as hypertension and coronary artery disease[135]. Diabetic patients may develop a diabetic heart disease (DHD) in which progresses with cardiac hypertrophy where the thickness of the left ventricular wall is increased and caused diastolic dysfunctions and other abnormalities[2]. Myocardial dysfunctions and impaired coronary perfusions in DHD are dependent pathologies associated with endothelial dysfunction initiated by diabetes[136]. Previous studies had showed that T2DM disrupts mitochondrial proteomic associated with protein import efficiency, which triggers mitochondrial dysfunction in diabetic patients leading to heart problems[4]. Further studies need to be explored in order to understand the causes of DHD; for instance, the development of an organ-on-a-chip can be established to construct experiments for deficiency of signaling pathways, drugs screening through systemic interactions by interconnecting different organs such as the pancreas and the heart or other organs affected by diabetes[137,138]. In addition, organ-on-a-chip can help to develop devices with sensors that can read glucose levels or increased proteins levels in the heart that may trigger heart failure; moreover, these state-of-the-art devices can also help to manage skin wound in risk of bacterial infections on those diabetic patients[9,139,140]. Status of stem

cell therapies and bioprinting in tissue repair and regeneration are shown in Table 3[141-181].

For T1DM

Besides providing for a constant source of β-cells, for serving therapeutic benefits in T1DM there is a need for a protective shell, which can house the newly regenerated β-cells while preventing antibodies from destroying them, thereby retaining their functionality.

Although T1MD has been treated by the transplantation of islets of Langerhans into the pancreas, it has necessitated the need to administer immunosuppressive drugs to the patients[182]. Since the side effects of these drugs have not been understood completely, cell transplantation therapy without the use of immunosuppressive drugs is preferred. Bioartificial pancreas has been fabricated by the encapsulation of islet cells within a semi-permeable membrane for the resolution of this issue[182]. Prior research has reported that these models function well with small animal models, but their clinical outcome on human patients remains to be studied further[182].

Scaffold-free tissue strands, expressing high levels of insulin, were microfabricated for extrusion based bioprinting by Akkouch *et al*[183]. These tissue strands were composed of rat fibroblasts and mouse insulinoma TC-3 β cells in the core and shell, respectively and were developed for scale up tissue engineering purposes[183].

Microscale organoids in which heterocellular aggregates possessed organ-like functions, have been successfully generated *in vitro* for pancreatic tissues by Greggio *et al*[184]. Efficient expansion of dissociated mouse embryonic pancreatic progenitors was enabled by establishing three-dimensional culture conditions in Matrigel[184]. Hollow spheres, composed of pancreatic progenitors, or complex organoids spontaneously undergoing pancreatic morphogenesis and differentiation, were generated by the manipulation of the medium composition[184]

Hiscox *et al*[185] successfully developed a tissue engineered pre-vascularized pancreatic encapsulating device (PPED) using collagen gels. It was observed that isolated islets that were placed in collagen gels exhibited fourfold more insulin release than islets not in collagen. Subsequently, a sandwich comprised of two layers of pre-vascularized collagen gels around a central collagen gel containing islets was also developed and implanted. *In vitro* characterization of the islets showed that islets were functional and responded to glucose stimulation[185]. Insulin and the presence of intra-islet endothelial cells were detected by performing immunohistochemical analysis. The results of the study indicated that PPED was able to enhance the islet survival by supporting islet viability and maintaining intra-islet endothelial cell structures[185]. Bloch *et al*[186] developed a technology to overcome the immunoisolation of pancreatic islets that leads to severe cell hypoxia and dysfunction. A thermophylic strain of the unicellular alga Chlorella was used as a natural photosynthetic oxygen generator to supply oxygen to the islets encapsulated in alginate[186]. The results of the study indicated that photosynthetic-dependent oxygen generation induced higher glucose-stimulated insulin response when compared to normoxic perfusion[186].

CONCLUSION

The ever-rising global burden of diabetes and its related complications is predicted to affect about 650 million by 2040 and is a major burden on our economy (American Heart Association). Diabetes mellitus is believed to be the underlying cause of functional and structural changes in the myocardium, that manifests in the condition referred to as diabetic cardiomyopathy (DCM), and may lead to heart failure independent of underlying coronary heart disease[187]. Patients with T2DM are recognized to have an increased risk of cardiovascular morbidity and mortality as hyperglycemia deteriorates endogenous cardiac protection[188]. Although DCM results from various mechanisms including microvascular impairment, metabolic disturbance, subcellular component abnormalities, cardiac autonomic dysfunction, and a maladaptive immune response, the underlying pathogenesis is partially understood. But there are major discrepancies among animal and human studies that leaves an important gap in knowledge[189]. Insights into the pathophysiology of human DCM are critical to discovering standardized targeted therapies. Therefore, there is an urgent need to biofabricate human tissue-on-a-chip models that can serve as a basis for development of novel therapeutic approaches to cure or prevent DCM *in vivo*. Bioprinting is a promising recent technology, which is likely to play an influential role in regenerative medicine. Many technical challenges still need to be overcome including limitations in resolution, cell distribution, vascularization, and innervation. However, this technology is poised to alleviate the treatment limitations of end-stage

Table 3 Status of stem cell therapies and bioprinting in tissue repair and regeneration

Organs	Stem cell	Bioprinting
Heart	(1) Combination of Mesenchymal and c-kit (+) Cardiac stem cell[141]; and (2) Human embryonic stem cell–derived cardiomyocytes[142]	(1) 3D bioprinting approach for vascularized heart tissue engineering based on human umbilical vein endothelial cells and induced pluripotent stem cells-derived cardiomyocytes[143]; (2) 3D-printed patch composed of human cardiac-derived progenitor cells in a hyaluronic acid/gelatin (HA/gel) based matrix[144]; and (3) 3D endothelial bed was seeded with cardiomyocytes to generate aligned myocardium capable of spontaneous and synchronous contraction[145]
Blood vessels	(1) Endothelial cells derived from human embryonic stem cells[146]; and (2) Human Pluripotent Stem cells[147]	(1) Pluronic F127 was used as a sacrificial material for the formation of the vasculature through a multi-nozzle 3D bioprinting system[148]; and (2) Drop-on-demand bioprinting technique to generate *in vitro* blood vessel models[149]
Nerves	Mesenchymal stem cell[150,151]	(1) Novel technique for bioprinting of fibrin scaffolds by extruding fibrinogen solution into thrombin solution, utilizing hyaluronic acid (HA) and polyvinyl alcohol[152]; and Production of high-resolution 3D structures of polylactide-based materials via multi-photon polymerization and explores their use as neural tissue engineering scaffolds[153]
Eyes	(1) Embryonic stem cell[154]; and (2) Limbal stem-cell[155]	(1) Produced 3D cornea-mimicking tissues using human stem cells and laser-assisted bioprinting[156]; and (2) Physical and chemical signals through 3D-bioprinting of HA hydrogels and co-differentiation of retinal progenitor cells into photo receptors [157]
Kidneys	(1) Embryonic stem cell[158]; and (2) Human pluripotent stem cells[159,160]	Bioprinting method for creating 3D human renal proximal tubules *in vitro* that are fully embedded within an extracellular matrix[161]
Skin	Mesenchymal stem cells[102,162]	(1) Amniotic fluid-derived stem cells printed in a set of pressure-driven nozzles through hydrogel solutions[102]; (2) Novel bioink made of gelatin methacrylamide and collagen doped with tyrosinase is presented for the 3D bioprinting of living skin tissues[163]; and (3) 3D cell printing of *in vitro* stabilized skin model and *in vivo* pre-vascularized skin patch using tissue-specific extracellular matrix bioink[164]
Pancreas	(1) Embryonic stem cells[165]; and (2) Human embryonic stem cells[166,167]	(Not fully developed) reviews[168,169]
Brain	(1) Multipotent adult stem cells[170]; and (2) Endogenous neural stem cells[171]	(1) Method for fabricating human neural tissue by 3D printing human neural stem cells with a bioink, and subsequent gelation of the bioink for cell encapsulation[172]; and (2) 3D bioprinted glioma stem cell model, using modified porous gelatin/alginate/fibrinogen hydrogel that mimics the extracellular matrix[173]
Lungs	(1) Distal airway stem cell[174]; (2) Pluripotent stem cells[175]; and (3) Exogenous stem/progenitor cells[176]	Reviews[177,178]
Liver	(1) Mesenchymal stem cells[179]; and (2) Induced pluripotent stem cells-derived organ bud transplant[180]	(1) Human embryonic stem cells-derived hepatocyte-like cells were 3D printed using alginate hydrogel matrix[117]; (2) Development of a liver-on-a-chip platform for long-term culture of 3D human HepG2/C3A spheroids for drug toxicity assessment[104]; and (3) Liver tissue model conducive to hepatotoxicity testing was developed by bioprinting hepatic spheroids encapsulated in a hydrogel scaffold into a microfluidic device[181]

organ dysfunction and failure. These challenges can be addressed by using more sophisticated printing technologies. Another possibility for addressing these challenges is through the fabrication and characterization of more sophisticated bioinks that deliver the necessary cues for promoting cell survival and the desired differentiation.

Hinton *et al*[190] developed a novel 3D printing method using the freeform reversible embedding of suspended hydrogels. This novel printing process generates intricate structures that mimic the properties of native tissues found *in vivo*, including the structures found in bone and brain.

Human cardiac cells prepared from iPSCs are incredibly useful as tools for generating human models of heart disease to acquire an improved understanding of the underlying mechanisms, and for testing different drugs or other treatments[191,192]. They can also be used to help predict which patients might have toxic cardiac side effects from drugs for other diseases. Such an advancement in stem cell-based tissue engineering will enable building of physiologically relevant cardiac tissue for applications in drug discovery and will further provide the opportunity to create personalized *in vitro* models from cells derived from patients[193]. The use of stem cell therapies and bioprinting in clinical practice will continue to emerge in the upcoming years. The availability of disease specific iPSCs such as those derived from patients having T1DM and T2DM have a huge potential towards fabrication of disease specific human tissue-on-a-chip models that may be used to model disease progression *in vitro*[194].

The employment of stem cells for the treatment of diabetes is still at its infancy stage in spite of the magnificent strides that have been taken in the field of stem cell biology and research. The research that has been done over the past decade has established that insulin-producing cells can definitely be derived from stem cells. However, the entire potential of stem cells can be harnessed only upon the resolution of associated issues and hurdles that fall in the way. Some of the key issues that limit the further exploration of stem cells in clinical trials include exploration of stem cells in clinical trials includes safety concerns, formations of teratomas, transplantation issues and autoimmune response, and also ethical dilemmas posed by ESCs. Similarly, the problems associated with the scale up production, further hamper the application of adult stem cells and iPSCs, as a choice of therapeutic resources. The need to formulate newer methods for the differentiation and selection of completely functional β-cell is a priority. The regeneration of these cells can be made possible only by controlling the regulation of various factors. The scientific efforts of the past research have made it possible to generate insulin-secreting cells and have laid the foundation for future research to come up with solutions utilizing stem cells as therapeutic agents to alleviate diabetes.

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REVIEW

Current methods for the maturation of induced pluripotent stem cell-derived cardiomyocytes

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Abstract

Induced pluripotent stem cells (iPSCs) were first generated by Yamanaka and colleagues over a decade ago. Since then, iPSCs have been successfully differentiated into many distinct cell types, enabling tissue-, disease-, and patient-specific *in vitro* modelling. Cardiovascular disease is the greatest cause of mortality worldwide but encompasses rarer disorders of conduction and myocardial function for which a cellular model of study is ideal. Although methods to differentiate iPSCs into beating cardiomyocytes (iPSC-CMs) have recently been adequately optimized and commercialized, the resulting cells remain largely immature with regards to their structure and function, demonstrating fetal gene expression, disorganized morphology, reliance on predominantly glycolytic metabolism and contractile characteristics that differ from those of adult cardiomyocytes. As such, disease modelling using iPSC-CMs may be inaccurate and of limited utility. However, this limitation is widely recognized, and numerous groups have made substantial progress in addressing this problem. This review highlights successful methods that have been developed for the maturation of human iPSC-CMs using small molecules, environmental manipulation and 3-dimensional (3D) growth approaches.

Key words: Induced pluripotent stem cells; Induced pluripotent stem cell-derived cardiomyocytes; Regenerative medicine; Stem cell biology; Translational research

Core tip: Induced pluripotent stem cells are key for generating disease-, patient-, and tissue-specific *in vitro* models. As such, induced pluripotent stem cells differentiated into

cardiomyocytes offer a potential tool for the understanding of disease and the development of life-saving therapeutics. Currently, cardiomyocytes can be differentiated with high efficiency but remain immature in their structure and function. Maturation of these cells is possible using a variety of approaches and will allow for more accurate disease modeling.

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INTRODUCTION

The discovery of induced pluripotent stem cells (iPSCs) by Takahashi *et al*[1] launched a novel field of medicine. The ability to differentiate human iPSCs (hiPSCs) into various cell types allows for the generation of patient-, disease- and tissue-specific cells. These cells enable precise disease modelling, *in vitro* drug testing, and clinical regenerative medicine approaches[1,2]. After a decade of research, iPSCs can now be successfully differentiated into hepatocytes[3], cardiomyocytes[4,5], neural cells[6,7], adipocytes[8] and many other cell types[9].

Cardiovascular disease is the greatest cause of mortality worldwide[10]. As such, modelling these diseases *in vitro* is of paramount importance to advance our understanding of disease and allow the development of new drug therapies. Cardiomyocytes derived from human iPSCs (hiPSC-CMs) enable the creation of a patient-, heart-, and disease-specific *in vitro* model[5,11]. This is potentially most useful for the study of very rare cardiac disorders, including metabolic cardiomyopathies[12]. These hiPSC-CMs are remarkably powerful as they replicate the genome of the patient donor and allow characterization of various diseases and drugs in a non-invasive manner[2]. In addition, their ability to contract allows for characterization of contractility and can thus serve as an accurate and translatable cardiac drug model[13]. Recent studies have also showcased hiPSC-CMs' ability to successfully engraft in a host organism[14,15]. One published study used macaque monkeys as a model for cardiomyocyte transplant outcomes. Transplanting human embryonic pluripotent stem cell derived-cardiomyocytes (hEPSC-CMs) through an intra-myocardial injection allowed the cells to graft with the host. Once attached, these cells showed crucial electromechanical coupling with the host as demonstrated by echocardiography[15]. Similar regenerative medicine studies have also been performed using small (guinea pigs) and large (pigs) animal models[16,17].

Over the past few years, the efficiency of hiPSC-CM generation has been significantly improved. Methods involving modulation of the GSK and Wnt pathways using small-molecule inhibitors have been widely used[5,18]. In addition, use of BMP and Activin A, along with the Matrigel sandwich method have proven successful[18]. Commercial kits such as those from STEMCELL Technologies (Vancouver, BC, Canada) and ThermoFisher Scientific (Carlsbad, CA, United States) have also entered the market and provide researchers with increased reproducibility and the ease of simplified protocols (Figure 1). Traditionally, hiPSC-CM generation has been characterised through flow cytometry staining for Troponin T (TNNT2), a cardiac-specific protein, in addition to visual qualification of spontaneously beating cell clusters. Current protocols allow for the production of > 80%-90% TNNT2-positive hiPSC-CMs. This showcases the field's success in achieving high-purity cardiomyocyte differentiation[2]. Through the use of lactate metabolic selection, > 99% TNNT2-positive cells have been successfully derived[19]. The derivation of highly-purified cardiomyocyte populations represented an important step forward for the field of cardiac regenerative medicine.

Although hiPSC-CMs are now being produced with high efficiency, an important problem remains. The hiPSC-CMs generated with current protocols and commercial kits are qualitatively and quantitatively immature[2,20,21]. For example, in addition to immature calcium handling, hiPSC-CMs display immature ultrastructural and electrophysiological features, low expression of key maturation markers, and rely on glycolysis for their metabolism as opposed to fatty acid metabolism[2,20,22]. Immature cardiomyocytes have important differences when compared to adult cardiomyocytes and these differences may cause inaccurate disease modeling or drug testing and lead

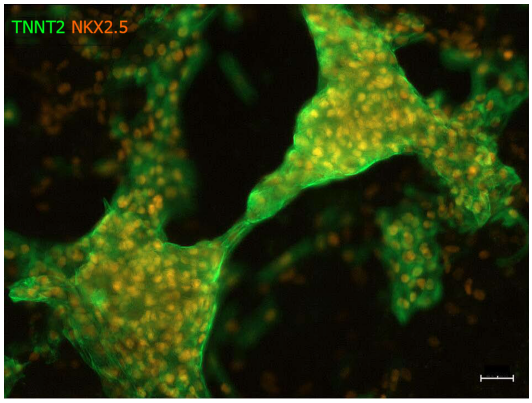

Figure 1 Human induced pluripotent stem cell-derived cardiomyocytes. Immunocytochemistry for TNNT2 cardiac marker (green) and NKX2.5 early-mesodermal marker (orange). HiPSCs were differentiated into hiPSC-CMs using ThermoFisher's Cardiomyocyte Differentiation kit. IPSCs were grown in a 6-well plate, differentiated into hiPSC-CMs for 14 d, fixed, stained, and then imaged using an epi-fluorescence microscope. Scale bar indicates 20 μm.

to unsuccessful clinical translation. For example, the effect of cardiac drugs on contractile characteristics may be inaccurate when using an immature model. However, given that hiPSC-CMs are being derived from pluripotent cells, it is not unexpected that the initial differentiated cells generated will be immature or fetal in their characteristics. It is therefore reasonable to expect that an additional maturation protocol (Figure 2) will be necessary to generate cells that truly reflect the *in vivo* tissue. As such, many research groups are currently focussing on methods to promote the maturation of hiPSC-CMs so that they are suitable for accurate disease-modeling and clinical applications. Methods evaluated to date include electrical stimulation, mechanical stimulation, modulation of carbon source, growth on various substrates, and the development of 3D culture conditions or organoids. Studies have also shown the positive effect of prolonged culture time on hiPSC-CMs[23,24]; however, culturing hiPSC-CMs for >90 d is neither time- nor cost-efficient and, given that these cells are usually cultured without antibiotics, remains a fraught enterprise. Therefore, other approaches must be used to create adult-like hiPSC-CMs within a reasonable time frame. Current protocols for hiPSC-CM production have failed to mature these cells due to a lack of knowledge regarding the mechanisms of heart maturation *in vivo*. At present, the field of cardiac regenerative medicine does not know the correct secretory factors, environmental cues, and external stimulation necessary to achieve proper adult-like cardiomyocytes. Maturing hiPSC-CMs is key to fully realizing the potential of these cells. Without proper maturation, hiPSC-CMs could cease to be clinically relevant. This review will examine the current methods for the maturation of iPSC-CM and suggest a way forward for the field.

ADULT CARDIOMYOCYTES *VS* hiPSC-CMs

Typically, adult cardiomyocytes differ from hiPSC-CMs in 4 important ways: (1) the expression of specific genes; (2) differing structural features; (3) altered metabolism; and (4) contractile function (Table 1)[22].

Gene expression

Adult cardiomyocytes express high levels of important structural genes such as *MYH7* (myosin heavy chain 7), *N2B* (cardiac titin), *cTNi* (troponin I), and *SERCA2* (sarcoplasmic reticulum ATPase)[22]. Adult cardiac heart tissues express high levels of genes such as *ITPR3* (inositol-1,4,5-triphosphate), *KCNH2* (potassium voltage-gated channel), *CAV3* (caveolin 3), and *RYR2* (ryanodine receptor 2)[2]. The importance of *CASQ2* (calsequestrin 2), *COX6A2* (cytochrome oxidase), *S100A1* (calcium binding protein 1), *SCN5A* (sodium voltage-gated channel alpha subunit 5) and *MYOM2/3* (myomesin-2/3) as markers of maturation has also been demonstrated[25]. In contrast, hiPSC-CMs display high levels of *MYH6* (myosin heavy chain 6) as opposed to *MYH7*, predominantly display the *N2A* isoform of cardiac titin instead of *N2B*[22] and have lower expression of other genes that are highly expressed in adult cardiomyocytes[26,27].

Structural features

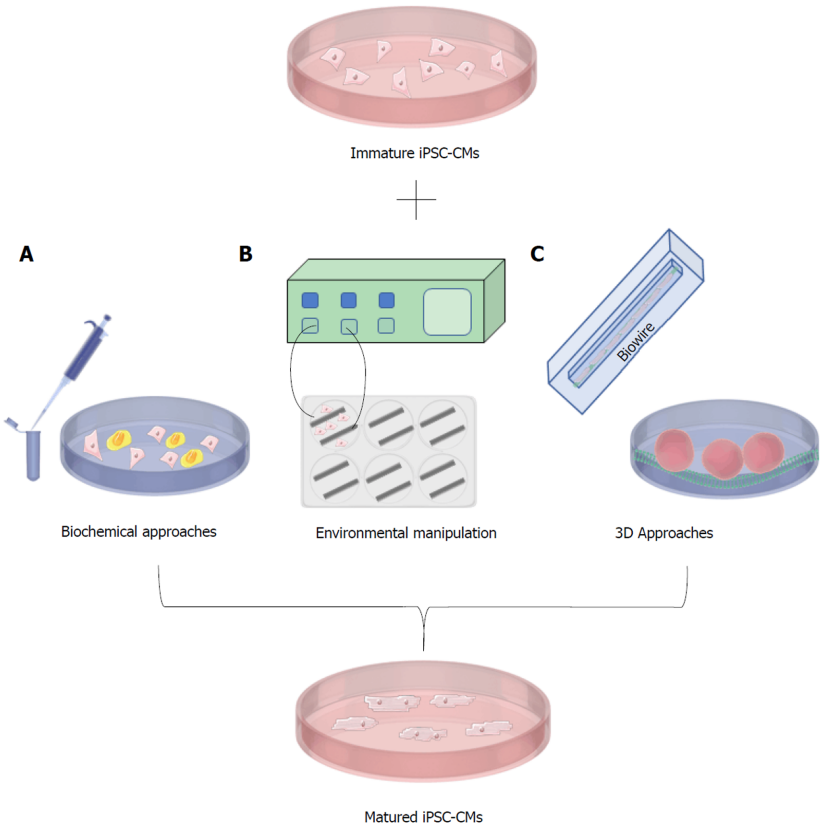

Figure 2 Methods for the maturation of induced pluripotent stem cell-derived cardiomyocytes. A: Biochemical approaches involving the use of small-molecules and hormones, along with co-incubation with human mesenchymal stem cells; B: Environmental manipulation illustrated through the use of electrical stimulation to mature iPSC-CMs; C: 3-dimensional approaches showcased by Biowire, and 3D cardiac organoid generation in ECM layered tissue-culture plates.

Structurally, adult cardiomyocytes display a high length-to-width ratio, may be bi-nucleated, and form sophisticated structures such as T-tubules and the sarcoplasmic reticulum within the sarcomere's Z-line[22]. T-tubules are significant due to their role in contraction propagation[28]. Absent or disrupted T-tubules have been implicated in heart failure in animal models[29]. Adult cardiomyocytes display Z-discs, I-, H-, A- and M- bands. In addition, adult cardiomyocytes have sarcomeres that are long (2.2 μm) and highly organized[22]. These cells also possess large numbers of mitochondria due to the heart's ceaseless energetic demands. Myocardial mitochondria tend to be evenly distributed throughout the cell and account for 20%-40% of cell size[22]. In contrast, immature hiPSC-CMs tend to be round, usually mono-nucleated, and the sarcomere is disorganized and shorter (1.6 μm). These cells also do not possess T-tubules and only have Z-discs and I- bands[22].

Metabolism

Metabolically, adult cardiomyocytes rely primarily on fatty acid oxidation as opposed to glycolysis for efficient energy production and have high levels of oxidative phosphorylation. In contrast, hiPSC-CMs mainly rely on glucose and lactate but do possess some capacity to metabolize fatty acids[2,22,27].

Contractile function

Adult cardiomyocytes tend to be more quiescent in terms of beating but generate greater force, upstroke and conduction velocities when stimulated[2,22]. In contrast, hiPSC-CMs have lower conduction and upstroke velocities but, due to an increase in the pacemaker current I_f, are still able to beat spontaneously[2,22].

BIOCHEMICAL APPROACHES FOR MATURATION

One approach for the maturation of hiPSC-CMs involves the manipulation of growth conditions through the addition of small molecules or changes in culture medium.

Table 1 Properties of adult cardiomyocytes *vs* currently generated Cardiomyocytes derived from human induced pluripotent stem cells

Properties	Adult cardiomyocytes	hiPSC-CMs
Gene expression	Higher: *MYH7, N2B, cTnI, SERCA2, ITPR3, CAV3, RYR2, CASQ2, COX6A2, S100A1, SCN5A, MYOM2/3* Lower: *MYH6, N2A*	Higher: *MYH6, N2A* Lower: *MYH7, N2B, cTnI, SERCA2, ITPR3, CAV3, RYR2, CASQ2, COX6A2, S100A1, SCN5A, MYOM2/3*
Structure	Elongated, high length to width ratio	Round, low length to width ratio
Sarcomere	Longer, organized	Shorter, unorganized
Types of nuclei	Some bi-nucleated	Mainly mono-nucleated
Banding	I-, H-, A-, M- and Z-discs	Z-discs and I-bands
T-tubules	Yes	No
Metabolism	Fatty acids, energy production through OXPHOS	Glucose, lactate, and fatty acids if present
Contractility	No spontaneous beating. Higher force, upstroke and conduction velocities	High spontaneous beating. Lower force, upstroke and conduction velocities

hiPSC-CMs: Cardiomyocytes derived from human induced pluripotent stem cells.

Tri-iodo-L-thyronine (T3), has shown promise in stimulating the maturation of hiPSC-CMs[30]. One study noted larger cell sizes and increased sarcomere length, in addition to higher contractile force and increased mitochondrial respiration capacity post-T3 treatment. Treated hiPSC-CMs also exhibited lower proliferative activity and improved calcium handling properties[30]. The authors of this study treated hiPSC-CMs with 20 ng/ml of T3 and noticed key morphological differences. Upon treatment, hiPSC-CMs became significantly less round and more elongated[30]. In addition, they found that cell size also increased post-T3 treatment. T3 treatment also resulted in higher expression of key maturation markers. The authors also showed that treated hiPSC-CMs exhibited increased contractile force as quantified through micropost arrays. Treated hiPSC-CMs exhibited a contractile force of 12.3 ± 0.7 nmol/L/cell while control cells were significantly lower at 7.5 ± 0.4 nmol/L/cell[30]. The mechanism of T3 in hiPSC-CM maturation is not completely understood; however, T3 has been shown to have an important role in cardiomyocyte differentiation through transcriptional regulation. Interestingly, blocking the action of T3 results in lower cardiomyocyte yield. It is hypothesized that downstream effects of T3 signaling may be responsible[30]. Studies suggest T3 by itself is insufficient in achieving maturation of hiPSC-CMs to an adult-like state. However, combining hormonal approaches with other strategies may be more successful. For example, treatment of hiPSC-CMs with both T3 and dexamethasone has shown success in furthering the maturational state of hiPSC-CMs[31]. When hiPSC-CMs were cultured with both chemicals for 15 days, an extensive T-tubule network was generated, a key indicator of adult-like cardiomyocytes as the extensions are crucial in contractility[31]. Many heart diseases result in defective T-tubule structures and therefore impaired contractility[29].

As current protocols for iPSC-CM generation result in cells that rely on glycolysis instead of fatty acid metabolism for energy, there has been recent emphasis on maturing hiPSC-CMs metabolically. One way of achieving this is through the use of glucose-free medium, forcing hiPSC-CMs to rely upon fatty acid metabolism. One study showcased how altering the metabolic state of hiPSC-CMs can induce increased maturation[32]. The authors exposed hiPSC-CMs to glucose-free medium containing insulin and fatty acids for three days. Doing so increased the sarcomere length significantly, showing the effect of altering metabolism on the structural features of the cell. In addition, this sarcomere length increase was correlated with improved electrophysiological characteristics[32]. Specifically, the upstroke velocity and duration of the action potential was increased in treated cells. Various maturation-related genes also displayed increased expression. A comparable study was carried out recently and displayed similar results. Correia *et al*[33] showed how the maturation state of hiPSC-CMs can be altered through the addition of galactose and fatty acids, accompanied by removal of glucose from the medium. Their experiments improved the metabolic, structural, and electrophysiological state of hiPSC-CMs. Immature hiPSC-CMs show remarkable flexibility in adapting to growth conditions. As such, incubating these cells with fatty acid-rich, glucose-free medium seems to be altering the transcriptional signature of these cells towards a more mature phenotype. As the immature hiPSC-CMs suddenly face glucose starvation, they may be pushed towards increasing transcription of genes key in metabolizing fatty acids in order to survive. As mentioned, fatty acid metabolism is characteristic of adult cardiomyocytes. Altering

the carbon source of hiPSC-CMs is easy to implement and these studies have shown its relative efficacy in maturing hiPSC-CMs.

Co-culturing hiPSC-CMs with other cell types has also been shown to further the maturation state of these cells[34]. Yoshida *et al*[34] describe matured hiPSC-CMs resulting from co-incubation with human mesenchymal stem cells hMSCs. As mesenchymal stem cells are reported to secrete factors key to the differentiation and electrical coupling of hiPSC-CMs, they sought to elucidate its effect on iPSC-CM maturity. The authors reported increased structural maturation through aligned A-, H-, and I- myofibrils, increased gap junction formation, increased energy production, and reduced reactive oxygen species production under stress[34]. This study then implicated VEGF, bFGF, SDF-1, and GM-CSF as secreted factors from hMSCs that are key in hiPSC-CM maturation. It is thought that secretion of these factors into hiPSC-CM cultures is able to induce maturation through upregulation of crucial adult cardiomyocyte gene *MYH7*.

The approaches mentioned in this section have all reported an increase in the maturation state of hiPSC-CMs. These approaches are simple and practical to implement but there are some key disadvantages. First, no study to date has shown complete maturation of hiPSC-CMs using only these methods. Although these methods further the state of maturation in these cells structurally, metabolically and electrophysiologically, they still do not create cells that fully recapitulate adult cardiomyocytes. These approaches may need to be combined with other more complex techniques such as electrical and mechanical stimulation.

ENVIRONMENTAL MANIPULATION

A hallmark of immature hiPSC-CMs is their electrical immaturity. These cells often display low expression of I_{Ks} potassium and I_{Na} sodium channels[2,22]. In addition, immature hiPSC-CMs spontaneously beat suggesting increased expression of the pacemaker current I_f[22]. To transition hiPSC-CMs into a mature electrical state, the use of electrical and/or mechanical stimulation is being extensively explored. Although potentially cost- and resource-prohibitive, these approaches may prove vital in the quest towards complete maturation of hiPSC-CMs. Cardiomyocytes can be subjected to various mechanical and electrical forces in an effort to mature them electrically. Previous studies elucidated the effects electrical pacing can have on cultured cardiomyocytes. In 2006, Brundel *et al*[35] showed the use of an *in vitro* system to model alterations in the contractility of cardiomyocytes by displaying how electrical pacing can induce tachycardia. Another study showed how stimulating canine cardiomyocytes through 24-h pacing can actually remodel the electrical features of the cells[36]. Furthermore, many studies have shown how electrical pacing can cause activation of L-type calcium channels, elevate intracellular Ca^{2+} levels and therefore stimulate increased contractility[37-39]. In hiPSC-CMs, authors of one study subjected these cells to both mechanical static stress (through maintenance of a fixed construct length) and mechanical static stress with electrical pacing conditions for 2 wks post-differentiation[40]. Their results were exciting as hiPSC-CMs exposed to both static stress and static stress with electrical conditioning experienced increased maturation. Both treatment groups experienced increased cell alignment, Frank-Starling force-length relationships, increased contractility, tensile stiffness and cell size[40]. These results display the success of mechanical and electrical stress in enhancing the maturation state of hiPSC-CMs[40].

One recent study showed the ability of heart muscle engineered through hiPSC-CMs to structurally and functionally mature through the use of passive stretch[41]. The authors created engineered tissue with the use of computational modelling and polydimethylsiloxane reservoirs to create passive stretch. They found that the tissue displaying a stretch of 7 mm resulted in the hiPSC-CMs showing increased expression of maturation genes involved in the troponin complexes along with potassium ion channels and T-tubule proteins[41]. This result suggests that passive stretch was able to induce both structural and functional changes in hiPSC-CMs. Other studies have also shown similar results suggesting that electrical and mechanical stimulation is an effective promoter of cardiomyocyte maturation[42-44].

Another promising approach involves the addition of conductive materials to the cell substrate or matrix. For example, the addition of polypyrrole chitosan (PPC) to create a composite hydrogel has shown great promise as a biomaterial capable of improving the conduction between clusters of cardiomyocytes. The authors of one study used calcium imaging to show how PPC improved electrical signal propagation between isolated rat cardiomyocytes and synchronized their contraction[45]. Although the effect of this hydrogel is yet to be evaluated in hiPSC-CMs, previously mentioned

literature has suggested that electrical stimulus is an important variable in achieving mature iPSC-CM populations.

As electrical and mechanical stimulation becomes more prevalent as a tool for hiPSC-CM maturation, private biotechnology companies have been developing electrical and mechanical devices commercially. One such device is C-Pace, a device created by IonOptix (Westwood, MA, United States). This device offers electrical stimulation and mechanical stretch through the use of a control interface and special plates outfitted with electrodes. The control interface allows for the manipulation of various current intensity and duration along with the force of mechanical stretch. This device can not only mature hiPSC-CMs functionally, but it can also induce arrhythmias and tachycardia for disease modelling. As mentioned, electrical and mechanical stimulation is not without its drawbacks. For one, it may prove cost prohibitive for many research groups. In addition, throughput is reduced as cells must be subjected to electrical pacing and/or mechanical stimulation for a certain period of time using specialized plates. However, these approaches seem to be important for the maturation of hiPSC-CMs and should be considered when developing a maturation protocol. The mechanism of hiPSC-CM maturation through the addition of mechanical and electrical cues is yet to be completely understood. However, it is hypothesized that conditional cues may upregulate the expression of key genes involved in establishing proper cardiomyocyte structure and contractility. For example, expression of calcium handling genes *SERCA2* and *RYR2* is increased following administration of static stress[40].

3D APPROACHES

Recently, 3D cardiomyocyte cultures, also known as organoids, have garnered great interest (Figure 3). The use of a multicellular 3D cultures potentially allow for higher accuracy in disease modelling and drug testing as 3D cardiomyocyte aggregates are closer to *in vivo* morphology[2,20,22]. Despite this, there have been significant challenges in developing 3D model systems. Some of these challenges include maintaining highly pure cardiomyocyte populations along with regulating clump/cluster size and providing adequate oxygen and nutrients. However, 3D culture also seems to improve the maturation state of hiPSC-CMs. In a recent study, 3D hiPSC-CMs had their transcriptome and metabolic status analysed[46]. The authors showed how 3D culture furthered the metabolic maturation of these cells and resulted in lower flux through glycolysis and increased oxidative phosphorylation[46]. Another study by the Radisic lab in Toronto described the development of a novel platform to mature hiPSC-CMs[47]. Through their Biowire device, the researchers were able to cultivate hiPSC-CMs in three dimensions. Differentiated hiPSC-CMs were seeded onto a wire substrate containing a template polydimethylsiloxane channel and collagen gels. After a week post-seeding, they noticed spontaneous contractions. They then exposed multiple cardiac wires to electrical stimuli and stimulated cells displayed improved calcium handling properties, increased myofibril organization, and higher conduction velocity[47]. Their results indicated the potential importance of an electrical stimulus combined with a 3D arrangement of hiPSC-CMs to induce maturation. In another study, a tissue-engineered cardiac patch was used to promote the maturation of hiPSC-CMs[48]. Differentiated cardiomyocytes became aligned through the use of passive tension and displayed higher conduction velocity and increased sarcomere length. Expression of various contractile genes such as *SERCA2* and *CASQ2* were also visibly increased[48]. Other 3D cardiomyocyte studies have displayed similar results[49,50]. These studies suggest a role for 3D culture in maturing hiPSC-CMs into adult-like states and improving disease models. 3D culture of hiPSC-CMs may be furthering maturation through provision of an environment closer to *in vivo* heart development. Culturing these cells in organoid formations could improve cell-cell contact and increase expression of various genes expressed in mature cardiomyocytes although the exact mechanism of maturation is yet to be elucidated. While promising, 3D hiPSC-CM models display some key disadvantages in disease modelling. First, many disease models require the use of single-cells to characterize disease phenotypes. Efficient dissociation and re-plating of 3D hiPSC-CMs is a known problem as many cells do not survive post-dissociation. This also poses a problem for potential clinical applications as typically, protocols involve the use of single cardiomyocytes for injection into a recipient animal myocardium [14,15]. Second, unless organoid cell numbers and aggregate size are not carefully optimized, drug testing may be inaccurate as organoids may not be exposed to the same dose of drugs. Further, routine cell sorting may be required to ensure the cellular homogeneity of cultured organoids. The recent development of tissue-culture plates such as

AggreWell (STEMCELL Technologies), and cardiomyocyte recovery/dissociation medium (STEMCELL Technologies) may prove useful in regulating cardiac organoid cell size and optimizing cell recovery; however, further research in this area must be done to definitively address these concerns.

CONCLUSION

Current protocols for the derivation of cardiomyocytes from iPSCs are highly efficient; however, hiPSC-CM culture conditions have not been adequately understood. Addition of various cytokines, environmental cues, and mechanical/electrical stimulation have yet to be optimized. As a result, current protocols result in cardiomyocytes that are most consistent in their properties with fetal cells which potentially limits their use for disease modelling and clinical translation of adult diseases. This review outlined several strategies for the maturation of hiPSC-CMs. The combination of several of these approaches may lead to the optimal maturation conditions.

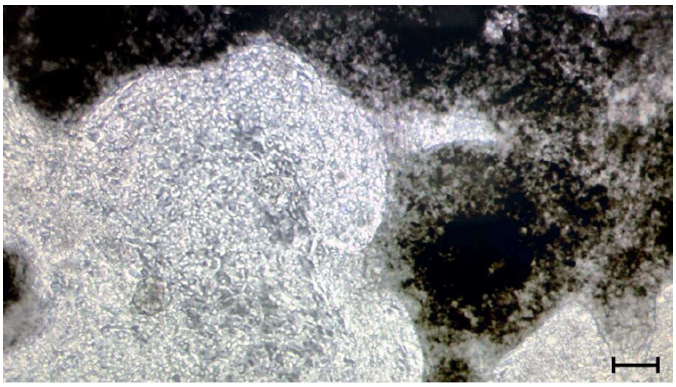

Figure 3 Induced pluripotent stem cell-derived cardiomyocytes in a 3-dimensional structure. Phase contrast image of a 3D formation of induced pluripotent stem cell-derived cardiomyocytes. Control hiPSCs were differentiated into hiPSC-CMs using STEMCELL Technologies cardiomyocyte differentiation kit. Image was taken at day 14 of differentiation. Scale bar indicates 40 μm.

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ORIGINAL ARTICLE

Basic Study

Regenerative potential of mouse embryonic stem cell-derived PDGFRα+ cardiac lineage committed cells in infarcted myocardium

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Author contributions: Hong SP and Song S contributed equally to this work; Hong SP and Song S performed the majority of experiments; Hong SP, Song S and Cho SW conceptualized the original idea, designed the experiments and analyzed the data; Hong SP and Lee S made myocardial infarction murine models; Park JH performed the transthoracic echocardiography and analyzed the data; Jo H, Kim HK and Han J coordinated and supervised the research; Hong SP, Song S and Cho SW wrote the paper.

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Abstract

BACKGROUND

Pluripotent stem cell-derived cardiomyocytes (CMs) have become one of the most attractive cellular resources for cell-based therapy to rescue damaged cardiac tissue.

AIM

We investigated the regenerative potential of mouse embryonic stem cell (ESC)-derived platelet-derived growth factor receptor-α (PDGFRα)$^+$ cardiac lineage-committed cells (CLCs), which have a proliferative capacity but are in a morphologically and functionally immature state compared with differentiated CMs.

METHODS

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We induced mouse ESCs into PDGFRα$^+$ CLCs and αMHC$^+$ CMs using a combination of the small molecule cyclosporin A, the rho-associated coiled-coil kinase inhibitor Y27632, the antioxidant Trolox, and the ALK5 inhibitor EW7197. We implanted PDGFRα$^+$ CLCs and differentiated αMHC$^+$ CMs into a myocardial infarction (MI) murine model and performed functional analysis using transthoracic echocardiography (TTE) and histologic analysis.

RESULTS

Compared with the untreated MI hearts, the anterior and septal regional wall motion and systolic functional parameters were notably and similarly improved in the MI hearts implanted with PDGFRα$^+$ CLCs and αMHC$^+$ CMs based on TTE. In histologic analysis, the untreated MI hearts contained a thinner ventricular wall than did the controls, while the ventricular walls of MI hearts implanted with PDGFRα$^+$ CLCs and αMHC$^+$ CMs were similarly thicker compared with that of the untreated MI hearts. Furthermore, implanted PDGFRα$^+$ CLCs aligned and integrated with host CMs and were mostly differentiated into α-actinin$^+$ CMs, and they did not convert into CD31$^+$ endothelial cells or αSMA$^+$ mural cells.

CONCLUSION

PDGFRα$^+$ CLCs from mouse ESCs exhibiting proliferative capacity showed a regenerative effect in infarcted myocardium. Therefore, mouse ESC-derived PDGFRα$^+$ CLCs may represent a potential cellular resource for cardiac regeneration.

Key words: Pluripotent stem cell; Embryonic stem cell; Cardiomyocyte; Myocardial infarction; Regeneration

Core tip: We demonstrated that mouse embryonic stem cell-derived platelet-derived growth factor receptor-α$^+$ cardiac lineage-committed cells have proliferative capacity but are in a morphologically and functionally immature state compared with differentiated cardiomyocytes; these cells exerted a regenerative effect on infarcted myocardium.

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INTRODUCTION

Myocardial infarction (MI) and heart failure are the most common causes of death in patients with cardiovascular disease[1]. Despite remarkable advances in therapeutic strategies for heart failure, such as novel drugs, ventricular assist device implantation, and heart transplantation, the burden of the disease remains high. Cardiac regeneration using stem cell therapy is an attractive therapeutic strategy to rescue damaged cardiac tissue[2]. Among stem cell populations, pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), exhibit a higher efficacy in cardiomyocyte induction and expansion rate compared with adult stem cells[2]. Indeed, previous large numbers of reports demonstrated functional improvement of damaged myocardium in murine, rodent, and porcine MI models that received PSC-derived cardiomyocytes (CMs)[2-4].

However, the proliferative capacity of PSC-derived CMs is decreased after beating and terminal differentiation[5]. Furthermore, there is no definite surface marker of differentiated PSC-derived CMs to facilitate purification[6]. Recently, several studies have been conducted to identify a novel marker for cardiac progenitor or cardiac lineage-committed cells (CLCs), which are intermediate-stage cells between mesodermal cells and differentiated CMs with proliferative capacity[7-10]. Our group previously established a novel class of cells from PSCs-platelet-derived growth factor receptor-α (PDGFRα)$^+$ CLCs-induced using a combination of four specific modulators:

the mitochondrial permeability transition pore inhibitor cyclosporin A (CsA), the ROCK inhibitor Y27632, the antioxidant Trolox, and the activin A receptor type II-like kinase (ALK5) inhibitor EW7197 (collectively referred to here as CsAYTE)[11]. This novel population of actively proliferating cells is cardiac lineage-committed but in a morphologically and functionally immature state compared with differentiated CMs[11]. In the present study, we investigated the regenerative potential of mouse ESC-derived PDGFRα$^+$ CLCs in a murine MI model and compared their efficacy with differentiated CMs.

MATERIALS AND METHODS

Mouse ESCs and OP9 cell culture

EMG7 mouse ESCs, which have an αMHC promoter-driven enhanced *GFP* gene, E14Tg2a ESCs, and OP9 cells were generated as described previously[12-14] and transferred to KAIST.

Generation of EMG7 mouse ESCs expressing tdTomato fluorescence

Lentiviruses were generated by transfecting FUtdTW (Addgene plasmid 22478)[15] with pMD2.G (Addgene plasmid 12259), pMDLg/pRRE (Addgene plasmid 12251) and pRSV-Rev (Addgene plasmid 12253)[16] in 293T cells using jetPEI (Polypus-transfection). Supernatants were collected 48 h after transfection, filtered through a 0.45 μm filter, and concentrated by Lenti-X concentrator (Clontech). Viral particles were resuspended in ESC medium with 4 mg/mL polybrene. EMG7 mouse ESCs were incubated in this medium for 24 h. Selection of ESCs was performed by FACS sorting.

Induction of mouse ESC-derived mesodermal precursor cells and CLCs

For the induction of Flk1$^+$ mesodermal precursor cells (MPCs), ESCs were cultured without leukemia inhibitory factor (LIF, Millipore) and plated on a 0.1% gelatin-coated dish at a cell density between 1×10^3 and 1.5×10^3 cells cm^2 in the differentiation medium, which is αMEM (Invitrogen) containing 10% fetal bovine serum (FBS, Welgene), 0.1 mmol/L of 2-mercaptoethanol (Invitrogen), 2 mmol/L of L-glutamine (Invitrogen) and 50 U/mL of penicillin-streptomycin (Invitrogen). Medium was changed every other day for 4.5 d. At day 4.5, differentiated ESCs were harvested with 0.25% trypsin-EDTA (Invitrogen), and antigen retrieval was performed in the differentiation medium for 30 min in an incubator. Then, cells were washed using 2% FBS in phosphate buffered saline (PBS) and incubated with biotinconjugated anti–mouse Flk1 antibody (clone AVAS12a1, eBioscience) and anti-streptavidin MicroBeads (Miltenyi Biotec). Flk1$^+$ MPCs were sorted by AutoMACS Pro Separator (Miltenyi Biotec). For induction of CLCs, sorted Flk1$^+$ MPCs were plated onto the mitomycin C (AG Scientific)-treated confluent OP9 cells at a density of 5-10 × 10^3 cells cm^2 in the medium containing 3 μg/mL of CsA, 10 μmol/L of Y27632, 400 μmol/L of Trolox, and 1 μg/mL of EW7197 (CsAYTE)[11,17]. The medium was refreshed every other day. Live images of differentiation process of CLCs and CMs were obtained using Axiovert 200M microscope (Carl Zeiss) equipped with AxioCam MRm (Carl Zeiss). Phase contrast images including beating CMs were obtained using an Infinity X digital camera and DpxView LE software (DeltaPix).

Flow cytometry analysis and cell sorting

The cells were harvested with 0.25% trypsin-EDTA or dissociation buffer (Invitrogen). To analyze live cells, antigen retrieval was performed in the differentiation medium for 30 min in an incubator and the cells were incubated for 20 min with the following antibodies: Allophycocyanin-conjugated anti–mouse PDGFRα (eBioscience, 17-1401, clone APA5, 1:100) and phycoerythrin/Cy7-conjugated anti–mouse Flk1 (BioLegend, 136414, clone AVAS12a1, 1:50). In live cell analysis and sorting, dead cells were excluded using 4,6-diamidino-2-phenylindole (DAPI, Sigma, D8417, 1:1000), and OP9 cells were excluded from Flk1$^+$ MPC by gating in flow cytometry. The differentiated CMs were sorted using αMHC-GFP. Analyses and sorting were performed by FACS Aria II (Beckton Dickinson). Data were analyzed using FlowJo Version 7.5.4 software (TreeStar).

Animals

Twenty eight male 9-wk-old BALB/c nude mice were kept in the specific pathogen free before the experiment under a 12:12 h light/dark cycle with lights on at 8:00 AM. They were deprived of food for 18 h but permitted water ad libitum before surgery. Animal care and experimental procedures were performed to conform the NIH guidelines (Guide for the care and use of laboratory animals) and approved by the

Animal Care Committee of KAIST (KA2013-40).

Preparation of acute MI model in mouse and cell transplantation

All mice were anesthetized through an intraperitoneal injection of a combination of anesthetics (80 mg/kg ketamine, 12 mg/kg xylazine) before any procedures. After intubation, the mice were ventilated with room air (SomnoSuiteTM, Kent scientific). MI was induced by exposing the heart by left thoracotomy and permanently ligating the proximal portion of left anterior descending coronary artery with an 8-0 prolene thread under respiratory support. After ligating the proximal portion of left anterior descending coronary artery, infarction of the anterior wall of left ventricle was confirmed in each mouse by the presence of a pale anterior wall and myocardial hypokinesis. Immediately after ligation of coronary artery and the confirmation of infarction, 100 μL PBS containing 1 x 10^6 PDGFRα$^+$ CLCs or αMHC$^+$ CMs were intramyocardially injected with a 31-gauge (0.25 mm) insulin syringe into the 3 different sites along the borderline of the infarcted area.

Transthoracic echocardiographic analysis

Transthoracic echocardiography (TTE) studies were performed (VIVID 7 dimension system, General Electric-Vingmed Ultrasound) 15 d after MI surgery and cell implantation. Images were obtained using an i13L transducer (5.3-14.0 MHz, GE Healthcare) with high temporal and spatial resolution. Two-dimensionally targeted M-mode parameters were measured at a level of papillary muscle in parasternal short axis view during 6 consecutive cardiac beats. All measurements were performed in a blind fashion according to the guidelines of American Society for Echocardiography.

Histologic and morphometric analyses

Before sacrifice, mice were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). For hematoxylin and eosin (H and E) staining, samples were fixed overnight with 4% paraformaldehyde and embedded in paraffin after tissue processing. For immunofluorescence staining, samples were fixed in 4% paraformaldehyde, dehydrated in 20% sucrose solution overnight, and embedded in tissue freezing medium (Leica). Samples were blocked with 5% goat (or donkey) serum in 0.01% Trition X-100 in PBS and then incubated overnight at 4 °C with the following primary antibodies: Mouse anti-α-actinin monoclonal antibody (Sigma Aldrich, A7811, clone EA-53, 1:100) or rabbit anti-α-actinin polyclonal antibody (Abcam, ab68167, clone EP2529Y, 1:100), rabbit anti-Ki-67 polyclonal antibody (Abcam, ab15580, 1:200), mouse anti-α-SMA monoclonal antibody (Sigma Aldrich, A2547, clone 1A4, 1:500), hamster anti-CD31 monoclonal antibody (Millipore, MAB1398Z, clone 2H8, 1:400), and rabbit anti-GFP polyclonal antibody (Millipore, AB3080, 1:200). After several washes, the samples were incubated for 2 h at RT with the following secondary antibodies: Cy5-conjugated anti-mouse IgG (Jackson ImmunoResearch, 715-175-150, 1:1000) and Cy3-, Cy5-, FITC-conjugated anti-rabbit IgG antibodies (Jackson ImmunoResearch, 711-165-152, 711-175-152, 711-095-152, 1:1000). Then the samples were mounted with fluorescent mounting medium (DAKO) and immunofluorescent images were acquired using a Zeiss LSM780 confocal microscope (Carl Zeiss). To calculate capillary density, number of CD31$^+$ endothelial cells was counted per random 0.5 mm^2 area in the infarcted myocardium at 15 d after cell implantation. To analyze the regenerative effects of host myocardium, number of Ki-67$^+$/α-actinin$^+$ CMs was counted per 104 nuclei in the peri-infarcted area, ranged within 200 μm from infarcted region, at 3 and 15 d after cell implantation. Images were analyzed using ImageJ software (http://imagej.nih.gov/ij/, 1.47V, NIH, United States).

Statistical analysis

Values are presented as mean ± SD. For continuous data, statistical significance was determined with the Mann-Whitney *U* test between 2 groups and the Kruskal-Wallis test followed by Tukey's honest significant difference test with ranks or multiple-group comparison. Statistical analysis was performed with SAS 9.4 (SAS Institute Inc). Statistical significance was set at $P < 0.05$ or 0.01.

RESULTS

Implantation of PDGFRα$^+$ CLCs and αMHC$^+$ CMs equally improves contractile function and structure in infarcted heart

To investigate the regenerative potential of PDGFRα$^+$ CLCs and αMHC$^+$ CMs, cells were sorted, and approximately 1 × 10^6 of each were implanted into the left

ventricular myocardium after inducing acute MI. The results of the recipient groups were compared with those of MI hearts without implantation. Analyses were performed at 2 wk after implantation of cells (Figure 1A). To trace the implanted cells in the infarcted heart, we induced PDGFRα$^+$ CLCs from ESCs expressing tdTomato fluorescence (Figure 1B). As shown in Figure 1C, the implanted cells were mainly distributed along several myocardial cavities 1 h after implantation.

First, to evaluate the functional recovery of infarcted hearts after cell implantation, we performed TTE 14 d after implantation. Compared with that in untreated MI hearts, the anterior and septal regional wall motion was notably and similarly improved (see arrowheads in Figure 2A) in the MI hearts implanted with PDGFRα$^+$ CLCs (hereafter designated as MI+PDGFRα$^+$ CLCs) and αMHC$^+$ CMs (designated as MI+αMHC$^+$ CMs). Moreover, the left ventricular internal dimension during systole of both MI+PDGFRα$^+$ CLCs and MI+αMHC$^+$ CMs was approximately 12%–23% less compared with that of untreated MI hearts (Figure 2B). Both MI+PDGFRα$^+$ CLCs and MI+αMHC$^+$ CMs also showed significant and similar improvements in systolic functional parameters, which included an ejection fraction increased by 20.0/15.6% and fractional shortening increased by 9.5/7.2%, respectively, compared with those of untreated MI hearts (Figure 2C). All TTE parameters are summarized in Table 1. These findings indicate that the implantation of PDGFRα$^+$ CLCs and αMHC$^+$ CMs had similar beneficial effects in the functional recovery of acutely infarcted hearts. Next, to confirm whether the implanted cells were properly engrafted to the infarcted myocardium, we performed histologic analyses at 15 d after implantation. Overall, the gross sizes of MI+PDGFRα$^+$ CLCs and MI+αMHC$^+$ CMs were smaller than that of untreated MI hearts (Figure 2D). Hematoxylin and eosin staining showed that untreated MI hearts had a thinner ventricular wall (0.19 mm) than did controls, while the ventricular walls of MI+PDGFRα$^+$ CLCs and MI+αMHC$^+$ CMs were similarly thicker (0.47 mm and 0.39 mm, respectively) compared with that of untreated MI hearts (Figures 2E and F).

Integration, differentiation, proliferation, and survival of implanted PDGFRα$^+$ CLCs in the infarcted heart

Importantly, implanted PDGFRα$^+$ CLCs and αMHC$^+$ CMs were visible as tdTomato$^+$/α-MHC-GFP$^+$ cells aligned and integrated with host CMs (Figure 3A). Implanted PDGFRα$^+$ CLCs and αMHC$^+$ CMs were mostly differentiated into α-actinin$^+$ CMs, and they did not convert into CD31$^+$ endothelial cells or αSMA$^+$ mural cells (Figures 3B and C). Moreover, CD31$^+$ blood vessels in the infracted area increased by 2.1- and 1.8-fold in MI+PDGFRα$^+$ CLCs and MI+αMHC$^+$ CMs at day 15 after implantation (Figures 4A and B), while the numbers of Ki-67$^+$ CMs also transiently increased equally by 2.4-fold at day 3; no such increases were detected at day 15 in both groups (Figures 4C and D). Thus, in addition to integration of implanted MI+PDGFRα$^+$ CLCs and MI+αMHC$^+$ CMs into the host myocardium, paracrine effects of MI+PDGFRα$^+$ CLCs and MI+αMHC$^+$ CMs appeared to be involved in the functional recovery of acutely infarcted hearts. Both types of implanted cells persisted up to 60 d after implantation (Figure 4E), which was the longest observation period in this study.

DISCUSSION

In the present study, we demonstrated the regenerative potential of mouse ESC-derived PDGFRα$^+$ CLCs in a murine MI model. Implantation of PDGFRα$^+$ CLCs and αMHC$^+$ CMs equally improved the contractile function and structure in the infarcted heart. Notably, implanted PDGFRα$^+$ CLCs were well integrated with host CMs and mostly differentiated into CMs.

Various transcription factors and cell-surface markers of cardiac progenitors or CLCs have been identified in previous studies[6]. Our group developed PDGFRα$^+$ CLCs induced by CsAYTE, which significantly enhanced the commitment of mesodermal cells to CLCs; in addition, the PDGFRα$^+$ CLCs can spontaneously further differentiate into CMs without additional manipulation or stimulation under *in vitro* conditions[11]. However, there are few studies regarding the engraftment and regenerative potential of cardiac progenitors or CLCs compared with differentiated CMs after implantation under *in vivo* pathologic conditions[18,19]. Takeda *et al*[7] recently found that human iPSC-derived CM-fated progenitors from a subpopulation of kinase insert domain receptor (KDR)$^+$ and PDGFRα$^+$ cells express CD82[7]. Consistent with our findings, purified CD82$^+$ cells gave rise to CMs under both *in vitro* and *in vivo* conditions[7]. Interestingly, CD82$^+$ cells showed considerably greater engraftment than differentiated vascular cell adhesion molecule 1 (VCAM1)$^+$ CMs after transplantation

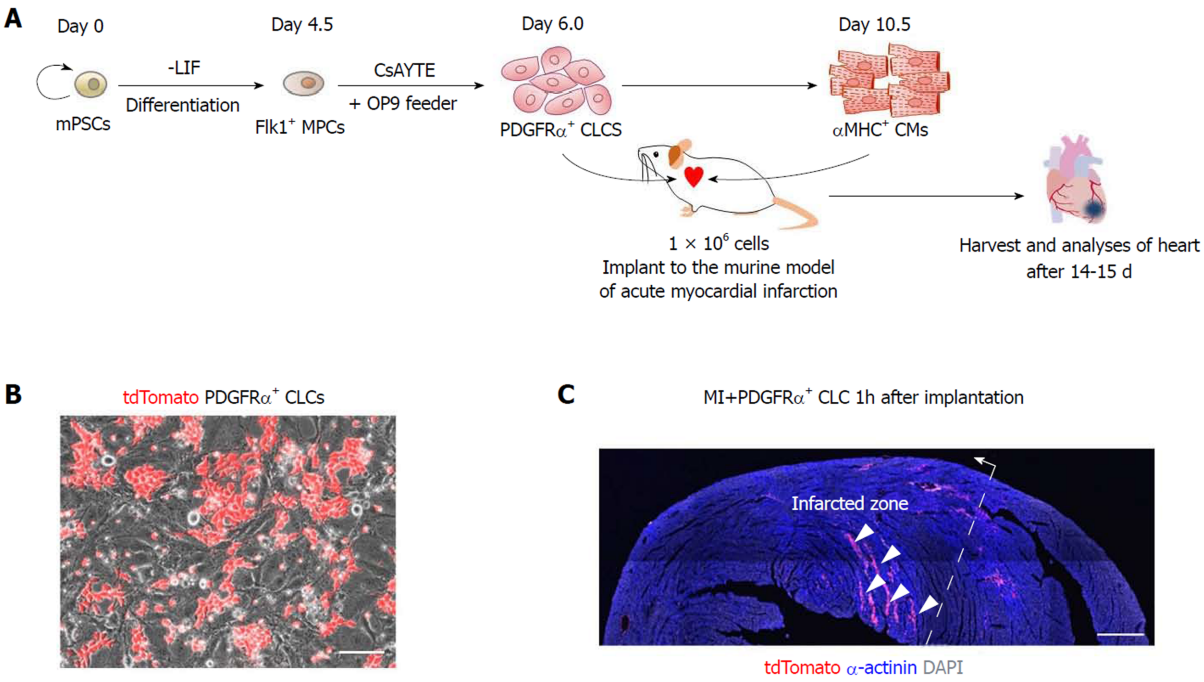

Figure 1 Implantations of platelet-derived growth factor receptor-α⁺ cardiac lineage-committed cells and αMHC⁺ cardiomyocytes in the acute myocardial infarction murine model. A: Experimental scheme of implanting either platelet-derived growth factor receptor-α (PDGFRα)⁺ cardiac lineage-committed cells (CLCs) or αMHC⁺ cardiomyocytes (CMs) into acute myocardial infarction (MI) murine model. Analyses were performed at 2 wk after implantation of approximately 1 × 10⁶ cells of PDGFRα⁺ CLCs or αMHC⁺ CMs into the left ventricular myocardium of acute MI murine model; B: Live cell image showing tdTomato⁺ cells during induction of PDGFRα⁺ CLCs from embryonic stem cells. Scale bars, 100 μm; C: Representative confocal image showing implanted tdTomato⁺ PDGFRα⁺ CLCs in the myocardial spaces (arrowheads) of the infarcted zone (dotted line and arrow), which was formed by ligation of coronary artery 1 h prior to the implantation. Scale bar, 500 μm. CLCs: Cardiac lineage-committed cells; CMs: Cardiomyocytes; PSCs: Pluripotent stem cells; ESCs: Embryonic stem cells; LIF: Leukemia inhibitory factor.

to the subrenal space[7]. These data indicated that the proliferative capacity of CLCs is higher than that of differentiated CMs under *in vivo* conditions. Furthermore, CD82⁺ cells primarily differentiated into CMs within infarcted hearts at approximately 95% efficiency; nevertheless, there were no data related to functional and structural recovery in the infarcted hearts[7]. The LIM-homeodomain transcription factor ISL1 is the most well-known marker of cardiac progenitors, and recent studies demonstrated that ISL1⁺ cardiac progenitors also exhibit regenerative potential in the infarcted heart[20,21]. Another developed strategy, direct reprogramming, was used to generate proliferative induced cardiac progenitors from fibroblasts with cardiac-specific transcription factors (Mesp1, Tbx5, Gata4, Nkx2.5, and Baf60c), and these reprogrammed cells were revealed to have regenerative potential in MI[22,23]. Collectively, the previous and current data provide compelling evidence that cardiac progenitors or CLCs are potential cellular resources for cardiac regeneration.

However, our data failed to demonstrate the superior regenerative effect of proliferative PDGFRα⁺ CLCs compared with differentiated αMHC⁺ CMs after implantation, consistent with a previous report[19]. Although PDGFRα⁺ CLCs exhibit more proliferative capacity than differentiated αMHC⁺ CMs, their expansion might be restricted owing to the limited space of the myocardium, especially in a small mouse model. Further experiments using large animal models, such as swine or non-human primates, might be necessary to confirm the regenerative effect of CLCs. In addition, the pathologic microenvironment of damaged heart might affect the proliferation and survival of implanted cells. Indeed, the previous and current data demonstrated that the engraftment of implanted CLCs and CMs was gradually decreased with time. Despite suboptimal engraftment and eventual death of the implanted cells in infarcted myocardium, the regenerative effect of implanted cells might result from differential paracrine effects[24]. Recent data demonstrated the significant upregulation of promigratory, proangiogenic, and antiapoptotic gene expression in the infarcted myocardium of groups treated with CMs compared with groups treated with PSCs and the controls[24]. Our data also revealed enhanced angiogenesis after implantation of PDGFRα⁺ CLCs and αMHC⁺ CMs. Therefore, the previous and current data suggested that not only direct integration but also the paracrine effect of implanted CLCs and CMs contributes to cardiac regeneration[24]. Further studies are needed to better understand the therapeutic mechanisms following transplantation of CLCs and to enhance engraftment.

Table 1 Echocardiographic parameters of Control, MI, MI+PDGFRα$^+$ CLCs, and MI+αMHC$^+$ CMs groups

Group	LVIDd (mm)	LVIDs (mm)	IVSd (mm)	IVSs (mm)	LVPWd (mm)	LVPWs (mm)	LVEDV (mL)	LVESV (mL)	LVSV (mL)	LVEF (%)	FS (%)
Control (n = 7)	4.46 ± 0.23	3.14 ± 0.14	0.76 ± 0.07	1.08 ± 0.05	0.76 ± 0.10	1.11 ± 0.07	0.22 ± 0.04	0.08 ± 0.01	0.14 ± 0.03	62.7 ± 2.52	29.2 ± 1.72
MI (n = 7)	5.57 ± 0.60	5.09 ± 0.54	0.67 ± 0.04	0.71 ± 0.07	0.74 ± 0.09	1.03 ± 0.17	0.42 ± 0.14	0.32 ± 0.10	0.10 ± 0.03	22.5 ± 2.91	8.61 ± 1.12
MI+PDGFRα$^+$ CLCs (n = 7)	4.96 ± 0.38	3.92^b ± 0.54	0.68 ± 0.10	0.87 ± 0.27	0.68 ± 0.06	0.99 ± 0.13	0.21^b ± 0.14	0.12^b ± 0.08	0.09 ± 0.06	42.4^b ± 4.38	18.1^b ± 2.54
MI+αMHC$^+$ CMs (n = 7)	5.15 ± 0.56	4.38 ± 0.60	0.65 ± 0.04	0.69 ± 0.04	0.74 ± 0.07	1.08 ± 0.12	0.28^a ± 0.14	0.18^b ± 0.10	0.10 ± 0.05	38.1^b ± 6.86	15.8^b ± 4.79

$^aP < 0.05$ *vs* MI.
$^bP < 0.01$ *vs* MI.
The parameters present as mean ± SD. LVIDd: Left ventricular internal diameter diastole; LVIDs: Left ventricular internal dimension systole; IVSd: Interventricular septal diastole; IVSs: Interventricular septal systole; LVPWd: Left ventricular posterior wall diameter diastole; LVPWs: Left ventricular posterior wall diameter systole; LVEDV: Left ventricular end diastolic volume; LVESV: Left ventricular end systolic volume; LVSV: Left ventricular stroke volume; LVEF: Left ventricular ejection fraction; FS: Fractional shortening; CLCs: Cardiac lineage-committed cells; CMs: Cardiomyocytes; MI: Myocardial infarction.

Proper electromechanical integration of PSC-derived CMs into host myocardium is crucial for preventing fatal arrhythmia after transplantation[1]. In a recent study, Chong *et al*[25] reported remuscularization of infarcted myocardium after injection of human ESC-derived CMs into non-human primate models of MI[26]. These grafts formed electromechanical junctions with the host myocardium and beat in synchrony, but ventricular arrhythmias were noted after transplantation[25]. Another recent study showed that monkey iPSC-derived CMs improved cardiac contractile function after transplantation into infarcted monkey hearts; nonetheless, the incidence of ventricular tachycardia was transiently but significantly increased[27]. In our study, we could not evaluate the occurrence of ventricular arrhythmia because of the technical difficulties associated with the mouse model. Therefore, further studies using large animal models might be necessary to confirm the arrhythmogenic effect of proliferating CLCs compared with differentiated CMs after transplantation into infarcted heart.

In conclusion, PDGFRα$^+$ CLCs served as the potential donor population for cardiac regeneration, and our findings provide conceptual and technical advances in stem cell therapy for cardiac regeneration.

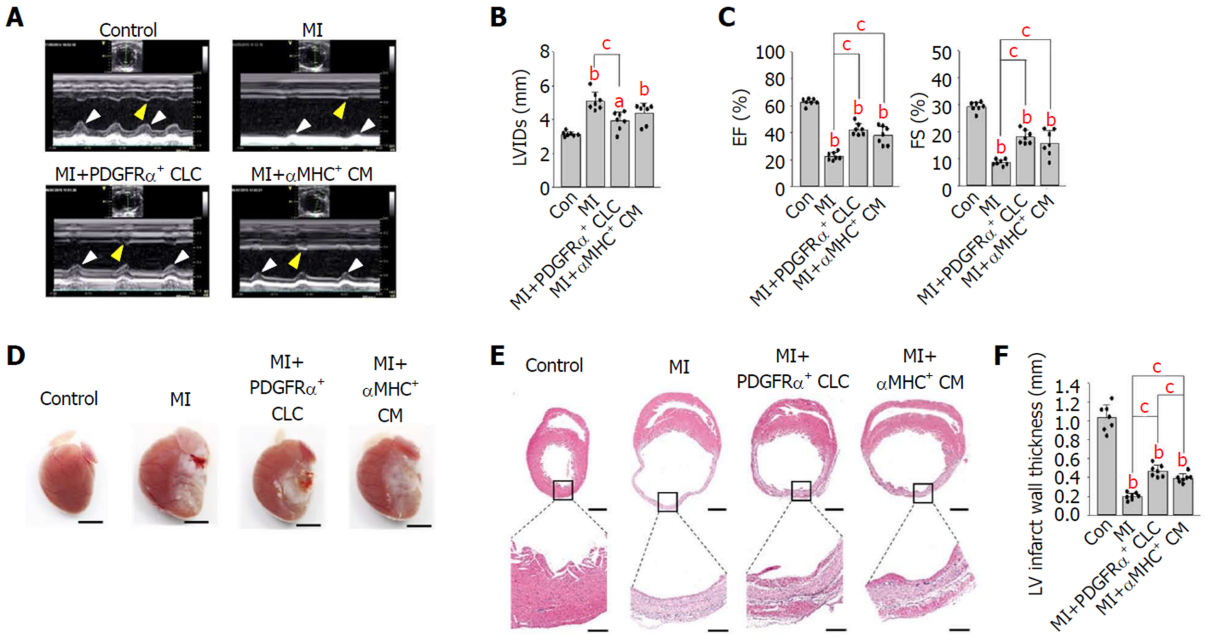

Figure 2 Implantations of platelet-derived growth factor receptor-α$^+$ cardiac lineage-committed cells and αMHC$^+$ cardiomyocytes equally improves contractile function and structure in the infarcted heart. A: Representative M-mode transthoracic echocardiography views of control, myocardial infarction (MI), MI+ platelet-derived growth factor receptor-α (PDGFRα)$^+$ cardiac lineage-committed cells (CLCs), and MI+αMHC$^+$ cardiomyocytes (CMs). Improved anterior (white arrowheads) and septal (yellow arrowheads) regional wall motion are observed in the left ventricles of MI+PDGFRα$^+$ CLCs and MI+αMHC$^+$ CMs; B and C: Quantifications of left ventricular internal dimension in systole (mm), ejection fraction (%) and fractional shortening (%). Each group, n = 7. $^aP < 0.05$ and $^bP < 0.01$ *vs* Con; $^cP < 0.01$ *vs* MI; D: Gross images of hearts in control, MI, MI+PDGFRα$^+$ CLCs, and MI+αMHC$^+$ CMs. Scale bars, 2.5 mm; E: H and E staining of mid-sectioned hearts of control, MI, MI+PDGFRα$^+$ CLCs, and MI+αMHC$^+$ CMs. Scale bars, 1 mm and 50 μm in the upper and lower panels, respectively; F: Quantifications of the thickness (mm) of left ventricle in the infarcted region. Each group, n = 7. $^bP < 0.01$ *vs* Con; $^cP < 0.01$ *vs* MI or MI+PDGFRα$^+$ CLCs. CLCs: Cardiac lineage-committed cells; CMs: Cardiomyocytes; MI: Myocardial infarction.

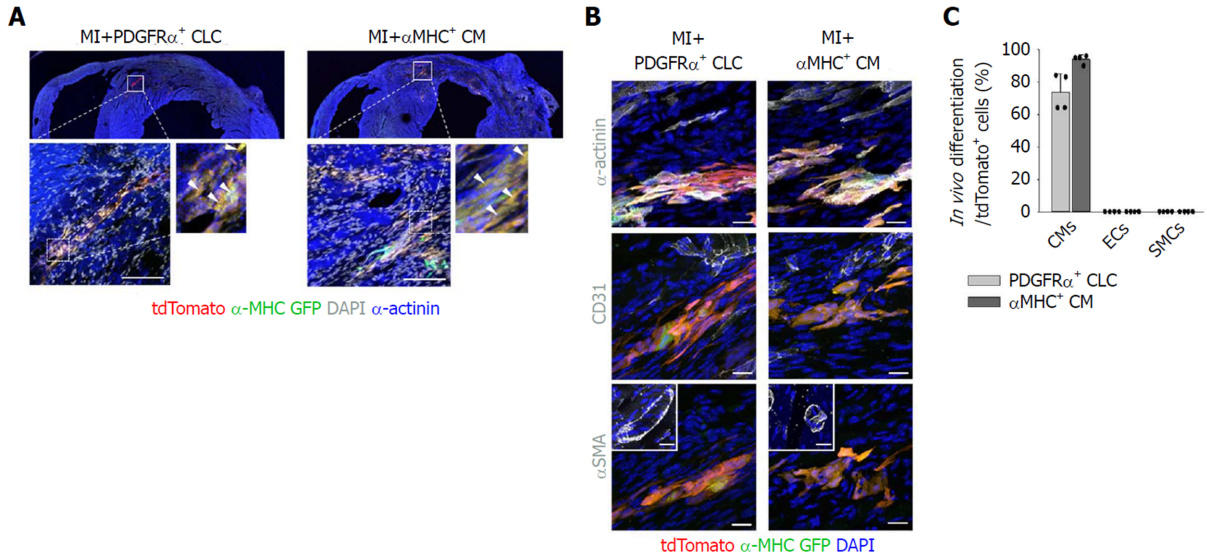

Figure 3 Integration and differentiation of implanted platelet-derived growth factor receptor-α$^+$ cardiac lineage-committed cells in the infarcted heart. A: tdTomato-tagged platelet-derived growth factor receptor-α (PDGFRα)$^+$ cardiac lineage-committed cells (CLCs) or αMHC$^+$ cardiomyocytes (CMs) were implanted into the infracted myocardium and integration was confirmed by immunostaining. tdTomato$^+$/α-MHC-GFP$^+$ cells (white arrowheads) are implanted PDGFRα$^+$ CLCs and αMHC$^+$ CMs. Scale bars, 100 μm; B: Representative confocal images showing differentiation of tdTomato$^+$ PDGFRα$^+$ CLCs and αMHC$^+$ CMs into cardiomyocytes 15 d after the implantation. αSMA-expressing cells were negative for tdTomato or α-MHC-GFP signal as shown in the inlet. Scale bars, 25 μm; C: Percentages of α-actinin$^+$ CMs, CD31 endothelial cells and αSMA$^+$ smooth muscle cells of the implanted PDGFRα$^+$ CLCs and αMHC$^+$ CMs. Each group, n = 4. Scale bars, 20 μm. CLCs: Cardiac lineage-committed cells; CMs: Cardiomyocytes; MI: Myocardial infarction; ECs: Endothelial cells; SMCs: Smooth muscle cells.

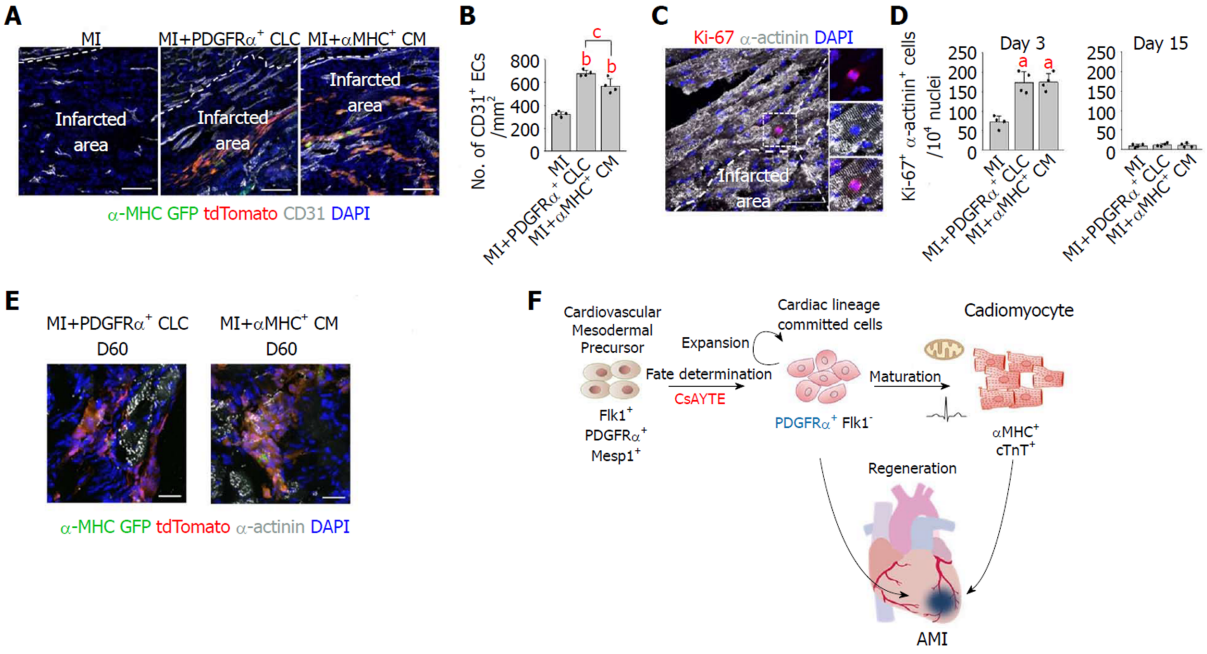

Figure 4 Proliferation and survival of implanted platelet-derived growth factor receptor-α+ cardiac lineage-committed cells in the infarcted heart. A: Representative confocal image of Ki-67+ host cardiomyocytes in the peri-infarcted region 3 d after cell injection. Dotted-lined rectangular region is magnified in right. Scale bar, 50 μm; B: Quantifications of Ki-67+α-actinin+ cardiomyocytes per 10^4 nuclei in the peri-infarcted region 3 and 15 d after the implantation. Each group, $n = 4$. $^aP < 0.05$ *vs* myocardial infarction (MI); C: Representative confocal images of revascularization within the infarcted areas 15 d after the implantation. Scale bars, 100 μm; D: Quantifications of capillary density (No. of CD31+ ECs/mm^2) within the infarcted areas. Each group, $n = 4$. $^bP < 0.01$ *vs* MI; $^cP < 0.01$ *vs* MI+ platelet-derived growth factor receptor-α (PDGFRα)+ cardiac lineage-committed cells (CLCs); E: Representative confocal images of tdTomato+ PDGFRα+ CLCs and αMHC+ CMs 60 d after the implantation. Three independent experiments showed similar findings. Scale bars, 20 μm; F: Schematic diagram illustrating the regenerative potential of PDGFRα+ CLCs in the infarcted heart. CLCs: Cardiac lineage-committed cells; CMs: Cardiomyocytes; MI: Myocardial infarction.

ARTICLE HIGHLIGHTS

Research background

Pluripotent stem cell (PSC)-derived cardiomyocytes (CMs) have become one of the most attractive cellular resources for cell-based therapy to rescue damaged cardiac tissue.

Research motivation

The proliferative capacity of PSC-derived CMs is decreased after beating and terminal differentiation. Furthermore, there is no definite surface marker of differentiated PSC-derived CMs to facilitate purification.

Research objectives

We investigated the regenerative potential of mouse embryonic stem cell-derived PDGFRα+ cardiac lineage-committed cells (CLCs) in a murine myocardial infarction (MI) model and compared their efficacy with differentiated CMs.

Research methods

We implanted platelet-derived growth factor receptor-α (PDGFRα)+ CLCs and differentiated αMHC+ CMs into a MI murine model and performed functional analysis using transthoracic echocardiography (TTE) and histologic analysis.

Research results

Compared with the untreated MI hearts, the anterior and septal regional wall motion and systolic functional parameters were notably and similarly improved in the MI hearts implanted with PDGFRα+ CLCs and αMHC+ CMs based on TTE. In histologic analysis, the untreated MI hearts contained a thinner ventricular wall than did the controls, while the ventricular walls of MI hearts implanted with PDGFRα+ CLCs and αMHC+ CMs were similarly thicker compared with that of the untreated MI hearts. Furthermore, implanted PDGFRα+ CLCs aligned and integrated with host CMs and were mostly differentiated into α-actinin+ CMs, and they did not convert into CD31+ endothelial cells or αSMA+ mural cells.

Research conclusions

PDGFRα+ CLCs from mouse ESCs exhibiting proliferative capacity showed a regenerative effect in infarcted myocardium. Therefore, mouse ESC-derived PDGFRα+ CLCs may represent a potential cellular resource for cardiac regeneration.

Research perspectives

PDGFRα$^+$ CLCs served as the potential donor population for cardiac regeneration, and our findings provide conceptual and technical advances in stem cell therapy for cardiac regeneration.

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