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CD34+ Very Small Embryonic-like Stem Cells or Induced Pluripotent Stem Cells for Cardiac Repair?

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Abstract

Current research in regenerative medicine is focused on finding pluripotent or multipotent cells with lower associated risks and fewer ethical problems when used as a treatment for patients. There are unresolved ethical and technical issues that hamper the clinical use of embryonic stem cells (ESCs). Even though induced pluripotent stem cells (iPSCs) avoid ethical issues, they present the same technical issues as ESCs, mainly related to their genomic dysregulation and epigenetic instability. In parallel, evidence has accumulated that adult bone marrow harbors different primitive cells which possess the ability to repair organs outside the hematopoietic system. It has been observed that in myocardial infarctions (MI), the injection of CD34+ cells close to the injured myocardial area can achieve a significant restoration of cardiac function, suggesting their direct and/or indirect involvement in the regeneration of heart tissue. This thought was sustained when pluripotent cells known as VSELs, which are able to differentiate and regenerate different organic tissues, were identified and isolated among CD34+ stem cells; this dismissed the presumption of the possible cell plasticity or de-differentiation. However, additional clinical studies are still to be carried out to further examine this. In the present review, we focused on the biological aspects of different stem cells to shed light on which most are promising, in the hope of improving the treatment of MI by regenerative medicine, once their manipulation becomes mastered.

Keywords: CD34+; VSELS; iPSCs; Regenerative Medicine; Acquired Heart Diseases; Cell Therapy; Myocardial Regeneration; Stem Cells; Heart Failure; Myocardial Infarction; Cardiovascular Disease

Introduction

The concept of regenerative medicine was conceived at the beginning of the 21st century, although hematopoietic stem cell transplantations-now included within this appellation-had already been performed for three decades to treat leukemias and other cancers. The goal of this new therapeutic approach is to inject stem/progenitor cells into diseased or damaged organs with the hope that they will form new healthy tissue in these organs, avoiding the need for further transplantation.

For a long time, embryonic stem cells (ESCs), which are immortalized pluripotent stem cells derived from an early embryo at the blastula stage, have been considered the ideal stem cells for applications in regenerative medicine, as they can give rise to cells from the three germ layers [1,2]. Their pluripotent differentiation potential theoretically enables them to generate any multipotent cell type, at least *in vitro* [3]. However, there are many problems associated with their use for clinical purposes. First, they are usually obtained by the destruction of unused embryos issued from parental projects, which is ethically highly controversial [4]. Besides ethical considerations, which reduces the possibility of their clinical use in most countries, their clinical use faces unresolved technical problems related to difficulties of achieving differentiation into fully functional cells at a large scale, the risk of teratoma or other tumor formation induced by these cells, and their genomic instability [5-7]. Furthermore, they give rise to immunogenic differentiated cells that might be rejected by a histo-incompatible recipient, requiring an immuno-suppressive treatment [8]. For these reasons, very few clinical trials using embryonic-derived progenitor cells have been authorized around the world, and most have achieved disappointing results. In the cardiology field, one group surgically sutured a fibrin patch bearing ESC-derived cardiovascular progenitors directly onto the epicardium of the ischemic myocardial area, in six patients scheduled for coronary artery bypass grafting (CABG) [9]. However, the confounding effect of the concomitant CABG, associated to the small sample size, precluded any meaningful conclusion regarding efficacy. Moreover, the intraoperative application of the patch and the alloimmunization, though clinically silent, developed by half of the patients, are limiting factors making this technology inappropriate for large scale clinical use. The therapeutic use of ESCs can now be considered to have clearly failed, despite the hype created by the media.

Over the same period of time, the successful application of hematopoietic stem cells (HSCs) in hematopoietic transplantation has encouraged clinicians to test, often empirically, adult stem cells in other therapeutic settings, mostly related to cardiac diseases [10]. In this indication, various types of cells have been employed, mainly isolated from bone marrow (BM), from peripheral blood (PB), or from fat tissue or cardiac biopsies. However, the cells used in most clinical trials performed during the two last decades were wrongly termed stem cells. For example, bone marrow mononuclear cells (BM-MNCs) are not stem cells but a mix of various cells of hematopoietic lineages at various stages of maturation, and their use in many clinical trials has failed to induce significant cardiac repair [11]. Mesenchymal stem cells (MSCs) are mainly stromal cell progenitor cells that do not have the properties required for clonal growth [12]; it is now well known that their weak beneficial effect is mainly due to the release of soluble factors or exosomes, is transient and does not induce myocardial tissue regeneration [13]. Adipose progenitor cells (APCs) issued from fat tissue are a variety of mesenchymal cells with similar properties, of which the only advantage is easy accessibility. The existence of cardiac stem cells, isolated from cardiac biopsies, is highly controversial [14]. Thus, CD34+ cells emerge as being the only "true" stem cell located in the BM.

CD34+ Cells

CD34+ cells were identified for the first time by Civin in 1984 [15]. CD34 is a membrane glycophosphoprotein that was discovered as a result of a strategy to develop antibodies that specifically recognize small subsets of human marrow cells, but not mature blood or lymphoid cells [16]. CD34 antibodies specifically detect approximately 1% of low density mononuclear cells from BM aspirates of normal donors, when there is less than 0.1% CD34 labeling of PB cells [17]. However, the intensity of CD34 expression on the cell's membrane is heterogeneous and correlates with the stage of cell immaturity/maturity, subdividing cells into CD34bright or CD34dim subgroups. The CD34bright are smaller and less dense than the CD34dim and correspond to the earliest progenitors, i.e., stem cells, representing at best one fifth of the total CD34+ cells, while the CD34dim are larger and denser and correspond to more committed progenitor cells, having lost their clonal growth properties [18]. CD34+ cells were considered for a long time as being only HSCs, giving rise to all hematopoietic lineages [16]. As they can be mobilized in large amounts from the bone marrow into the PB by hematopoietic growth factors (HGF), achieving approximately a two-logs cell enrichment, practice of PB-stem cell (PBSC) transplantation increased exponentially from the first attempts in the mid-1980s [19,20] up to totally replacing BM transplantation in hematological cancers [21].

From the mid -1990s, it was demonstrated that "true" HSCs, inducing and sustaining post-aplasia hematopoietic recovery in the long term, and characterized by a lack of the CD38 differentiation marker [17,22], only represent a small part (≈1%) of the total CD34^{+} cells, raising the question of what the remaining CD34^{bright} cells were. In 1997, Asahara *et al.* isolated a subpopulation of progenitor endothelial cells bearing the CD34 antigen in mice BM, which was capable of inducing neo-angiogenesis, thus demonstrating for the first time the existence of non-hematopoitic CD34^{+} cells [23]. In the following years it was progressively established that the CD34 antigen was also a marker of cardiac, liver, and osteoblastic progenitor cells [24-26]; each of these CD34^{+} subpopulations represented approximately 1% of the total CD34^{+} cells. In an attempt to explain this variety of CD34 subpopulations, several investigators proposed the hypothesis that HSCs could transdifferentiate into other lineages of progenitor cells, even those normally issued from another germ layer, opening the path for the transgressive concept of "cell plasticity" that persisted for years.

Apart from hematopoietic transplantation, CD34^{+} cells have been mainly employed in attempts to achieve cardiac repair after myocardial infarction (MI). Experimental and physiological data support such use of CD34^{+} stem cells in this indication. For example, CD34^{+} cells isolated from human PB after HGF mobilization stained positively for c-Troponin-T when transplanted directly into the scar of athymic rats with experimental MI, indicating they may also differentiate into cardiomyocytes [27]. In humans, endogenous CD34^{+} cells are released into the blood within the first hours following MI and for approximately one week, which seems to be a physiological response to limit ischemic scar formation [28].

We were the first to perform direct intra-cardiac delivery of large amounts of PB-CD34^{+} cells, previously purified by *in vitro* immune-selection, in patients suffering from bad-prognosis MI [29], and to further demonstrate the long-term benefit on regional heart structure and function of this breakthrough approach [24]. We also provided evidence that CD34^{bright} cells could differentiate both endothelial and cardiac progenitor cells after *in vitro* culture on an appropriate and proprietary medium we had developed [24]. Other investigators followed the same direction, using CD34^{+} cells purified and enriched by immuno-selection either from PB after mobilization [30] or BM aspirates [31], but with less significant results, due to variations in the numbers of cells delivered, the route of injection, and the stage of the ischemic disease, which are key factors for successful therapy [32].

The cardiac repair mechanisms of CD34^{+} cells are likely multi-faceted. Once activated by a complex blend of cardio-active chemokines secreted by the inflammatory scar [33,34], injected CD34^{+} cells may release soluble paracrine factors and exosomes that can enhance the proliferation of resident cardiomyocytes [35] or neo-angiogenesis [36], respectively, thus reducing fibrosis and avoiding remodeling effects. The scar chemokines also chemoattract CD34^{+} stem cells to the ischemic zone and induce their commitment along the endothelial and cardiac pathways [33], which is strongly dependent on changes that occur in myocardial stiffness after AMI [34]. Such commitment cannot occur under steady-state conditions but is crucial for the induction of cardiac tissue repair after ischemic disease.

Considering these data, it now seems that some, if not all, CD34^{+} cells might be pluripotent rather than multipotent stem cells, capable of giving rise to a large panel of organic tissues under specific stimulations. This would be a more realistic explanation for their role in organ repair than the romantic concept of cellular plasticity, which may need to be rejected.

Very Small Embryonic-Like Stem Cells (VSELs)

For the first time in 2006, Ratajczak, *et al.* [37] described a type of cells which have kept their embryonic capacities in the bone marrow of adult mice [37]. Indeed, these cells, known as very small embryonic-like stem cells (VSELs) have similar characteristics to ESCs and are believed to originate from primordial germ cells in the yolk sac, after which they migrate, escape specification into tissue-committed stem cells, retain their pluripotency and settle in different organs and persist throughout life (reviewed in [38]). Their existence was further demonstrated in humans by the same group and several others [39-42]. Interestingly, VSELs are phenotypically characterized as being CD34^{+}, CD133^{+} and/or CXCR4^{+}, but do not express lineages (Lin^{−}) and hematopoietic (CD45^{−}) markers [37,43]. They can be sorted and isolated on the basis of their phenotypic features and their small size (5 to 6 μm). They express embryonic pluripotent stem cell-specific markers, such as SSEA-4 and TRA-1-81, on their surface, and Oct-4, Nanog and Sox-2 transcription factors at the protein level. VSELs constitute a rare and homogeneous pluripotent fraction of CD34^{+} stem cells and quiescent that prevents them from over-proliferation and potential risk of teratoma formation. Their quiescence under the steady state is related to the expression of low levels of genes involved in proliferation and cell signaling, which become upregulated during cell activation. In addition to the bone marrow and peripheral blood, VSELs have also been identified in umbilical cord blood and other organs [44], diversifying their

source of collection and sampling. They can support vessel formation in vivo [45], and be specified to cardiomyocytes [46,47] neurons [48] and hematopoietic stem cells [49], both *in vitro* and in animal models.

Despite the huge reserves generated by this discovery, several laboratories were then able to characterize their biology and function when they were correctly isolated [41,42,50,51]. Populations similar or resembling VSELs have been described depending on the experimental strategies and diverse markers used for their isolation [52–54]. We observed that VSELs were positive for a pluripotent transcription factor, Nanog, isolated from umbilical cord blood. VSELs enclose heterogeneous cells expressing overlapping receptors; as such, a characterization of their biology is required [55]. We previously demonstrated that they circulate in very small numbers in peripheral blood under a steady state throughout life [56], but they can be mobilized in larger numbers by G-CSF from the BM into the PB [57]. Moreover, VSELs are actively mobilized from the bone marrow to the peripheral blood following stressful states such as myocardial infarction [58], in patients with leukemia [59] or stroke [60,61]. In Transwell chemotaxis assays, plasma collected from patients 6 h after AMI at hospital admission strongly chemo-attracted human bone marrow VSELs, suggesting that the AMI cardiac cells secrete specific chemoattractant growth factors [62].

Emerging evidence indicates that CD34+ VSELs could be at least in part responsible for the repairs and improvement of the cardiac function observed when using CD34+ cells in regenerative medicine after MI. Due to their properties, and as they are thought to be a reserve source of pluripotent CD34+ stem cells, their development could be used to ensure a safe, successful and efficient regenerative therapy for various non-curable diseases. Over the past several years, different reports indicated that in addition to HSCs, endothelial progenitor and mesenchymal stromal cells, VSELs are recruited to peripheral blood during MI, contributing to the repair of infarcted myocardium [63-65]. *In vitro* and in vivo studies have demonstrated through animal models that they are capable of regenerating several cell types, including cardiomyocytes and vascular endothelial cells, representing a serious alternative to other stem cell sources in cardiac regenerative trials. In acute experimental MI, in contrast to HSC CD45+, an intra-myocardial injection of VSELs is able to better attenuate left ventricular dysfunction and improve ejection fraction [46], as well as reduce myocardial hypertrophy [66]. A comparable effect was observed in chronic heart failure models [47]. Transplantation of VSELs-like MSCs triggered an improvement in cardiac function and heart remodeling in infarcted rats [67], similar to our data

recorded with an expanded CD34+ cell graft [68]. Zuba-Surma, et al. [69] observed in mice a concomitant increase of pluripotent markers (Oct-4, Nanog, Rex-1 and Dppa1) and VSELs in PB 48 h after MI induction, whereas this was not observed for HSCs [69]. An increased expression of pluripotency transcripts specific to VSELs concomitant improvement of cardiac markers secreted on PB was observed after treatment by the natural anti-oxidant molecule resveratrol, suggesting that its beneficial effect on heart function is associated with the activation of VSELs. These data support further study and development of VSELs with a view to their use in cardiac regeneration. Finally, interest in VSELs as a source of stem cells for regenerative applications increased once some clinical studies demonstrated their therapeutic potential in patients with chronic heart failure [66,70].

Before considering the use of VSELs in regenerative medicine, it is necessary to resolve the problem of their limited number by finding the means to control their quiescence [71], thereby stimulating their capacity for proliferation without affecting their pluripotency. However, in order to determine and establish how to make them proliferate in the presence of an appropriate support without feeders, many groups looked for conditions suitable for their proliferation. A positive proliferative was observed in response to their stimulation by specific growth factors and gonadal sex hormones (nicotinamide, FSH, LH, BMP-4, FGF-2 and KL) [72,73], as well as with UM171, a pyrimidoindole derivative known to be able to induce the self-renewal of hematopoietic stem cells [74]. This had a positive effect on the expansion and proliferation of different populations of VSELs, significantly increasing their number without affecting their ability to differentiate into specific organ cells [55]. Given the phenotype of VSELs and their unique characteristics, purified CD34+ cells are presumed to contain small numbers of CD45- cells, with only a small fraction containing VSELs, which explains the need for a large numbers of cells to have a significant effect on cardiac repair.

Induced Pluripotent Stem Cells (iPSCs)

Among other challenging approaches to prevent or even reverse the destruction of cardiac tissue, induced pluripotent stem cells (iPSCs) technology has generated a great interest due to its potential feasibility in cardiac regenerative medicine. These cells were developed by Yamanaka's research group in 2006 [75], based on the transduction of somatic mouse fibroblast with a cocktail of four genes (Oct4, Sox2, c-Myc and Klf4) that encode transcription factors governing pluripotency via integrating vectors (virus, lentivirus), resulting in the acquisition of the

morphology and growth properties of ESCs. Yamanaka's team and others have further demonstrated that human adult cells can also be reprogrammed to restore ESC characteristics by introducing the same or additional set of transcription factors [75,76]. Despite their ability to multiply indefinitely and to differentiate again in all cell types, they remain distinct from ESCs.

Avoiding any ethical issue, iPSCs constitute an exciting alternative source of pluripotent stem cells for disease modeling, pharmacological screening and cardiac cell therapy development. However, it has been demonstrated that low-passage of mouse iPSCs-derived by factor-based reprogramming harbors residual DNA methylation signatures, and maintains characteristics of their somatic tissue of origin as epigenetic memory (methylation) and genetic signature (that extends to miRNA), which may affect their lineage-specific differentiation capacity [77-79]. In addition, iPSCs generated with reprogramming factors via either integrating retroviruses or lentiviruses might cause insertional mutagenesis [80]. Furthermore, major issues regarding iPSCs have limited their clinical use, for example, their teratoma formation ability [81] resulting from their somatic cells of origin or occurring during the stepwise reprogramming process to iPSCs [82], and the risk of associated cancer formation linked to c-Myc and Klf4 known as potent oncogenes [83].

In order to increase the safety of iPSCs for clinical applications, human iPSCs technology has evolved through various non-integrative approaches, including reprogramming using episomal DNA [76,84], adenovirus [85], Sendai virus [86], Piggy-Bac transposon [87], a non-viral minicircle vector [88], use of recombinant proteins [89], or synthetically modified mRNAs [90] and micro RNAs [91]. All of these methods have made it possible to theoretically avoid the risk of insertional mutagenesis and genetic alterations [92].

Besides the hope generated by their use in the regeneration of the retina, iPSCs have aroused significant interest over the past decade in relation to cardiac repair, particularly in the treatment of myocardial infarction through regeneration. Ieda, *et al.* [93] were the first to describe a possible reprogramming of mouse fibroblasts into induced cardiomyocytes-like cells directly with the expression of three cardiogenic transcriptional factors (i.e., GATA-4, Mef2c and Tbx5), which may provide a source of cardiomyocytes for regenerative approaches [93]. However, only a small proportion of these converted cells display spontaneous contractions and express cardiomyocyte global gene expression profiles. Indeed, this technology was not sufficient for cardiac reprogramming in humans and needed other factors to be able to induce a cardiac-like phenotype [94,95].

The next challenge is to improve these findings and transfer the mouse direct reprogramming approach into humans, before their clinical application. To establish more efficient conditions for conversion of adult human fibroblasts to a cardiac phenotype, forced expression of cardiac transcription factors GATA-4, Hand2, TBX5, myocardin and Hand2 combined with muscle-specific microRNAs have shown that human fibroblasts can be reprogrammed to cardiac-like cells and displayed sarcomere-like structures and calcium transients; however, only a small subset of such cells exhibited spontaneous contractility [95]. Other strategies have been explored and revealed that an addition of Hand2 [96] and a constitutively active form of AKT to the identified cardiac transcription factors synergistically activated genome-wide cardiogenic stage-specific enhancers [97]. In addition, human fibroblasts could be converted into functional cardiomyocytes by a combination of nine small molecules, resulting in induced cardiomyocyte-like cells that uniformly contracted and resembled human cardiomyocytes in their transcriptome, epigenetic, and electrophysiological properties [98]. However, difficulties have been observed during the application of iPSCs in the heart field by the fact that all cells do not fully differentiate into cardiomyocytes and consequently are unable to restore massive loss of cardiac tissue responsible of fibrosis and scar formation [99].

Recent studies have shown that combining three human iPSCs-derived cardiac cell types in a three-dimensional micro-tissues could improve sarcomeric structures with T-tubules, enhance contractility and mitochondrial respiration, and promote electrical maturation associated to connexin 43 (CX43 gap junction) and intracellular cyclic AMP (cAMP) pathway activation [100]. It has been also demonstrated that sustained activation of AMP-activated protein kinase in human iPSC-derived cardiomyocytes via Sirtuin activation improved differentiation, leading to decreased acetylation of histones H3 (at Lys9 and 56) and H4 (at Lys16), and increased mRNA and protein expression of both TNNI3 and TNNT2 [101]. Given the problems with rejection that can arise during the use of iPSCs due to the need for MHC matching and immunosuppressive treatment [102], another debate has emerged regarding the use of autologous versus allogeneic iPSCs. The autologous setting implies a selection of cells specific to the patient, making their use lengthy, expensive, laborious and regulated. Indeed, generating single graft iPSCs from proper patient cells could take several months of preparation, which may be unrealistic for the treatment of diseases such as MI. In contrast, transplanted allogenic iPSCs-derived cardiomyocytes, in association with immunosuppressors, can survive

for up to 12 weeks in a macaque heart, but these animals presented ventricular tachycardia, which could be due to the immature state of the transplanted cells [103].

Although some reports have demonstrated that cardiomyocytes derived from iPSCs can be grafted into myocardium of animals and improve left ventricular function [104,105], in most cases iPSCs differentiate into immature cardiomyocytes with potential arrhythmic complications rather than to adult functional cardiomyocytes harboring their structures and gene expression profiles, particularly in larger animals [103,106,107]. A pilot study using allogeneic iPSCs-derived cardiomyocytes directly injected in the myocardium during a CABG operation has been recently launched in China [108], but is still in the recruitment process of five patients with ischemic heart disease. In addition,

other groups have reported that iPSCs do not directly contribute to the regeneration of cardiac tissues but are sources of growth factors that provide only a paracrine effect [109-111]. Recent studies using a mouse model of MI show that the transplantation of iPSCs can improve heart function via paracrine action [112]. In addition, iPSC-derived extracellular vesicles induce superior cardiac repair in vivo than cell transplantation, representing a safer alternative [113]. In 2018, the Japanese government approved a world-first study in which the investigators prefer to surgically implant degradable sheets of heart muscle tissues made from allogeneic iPSCs onto the external surface of the infarcted area as a source of growth factors, micro-vesicles, or exosomes, rather than integrating iPSCs-derived cardiomyocytes into the host cardiac tissue [114]. The first of the ten patients scheduled was treated at the beginning of 2020, but no data are available yet.
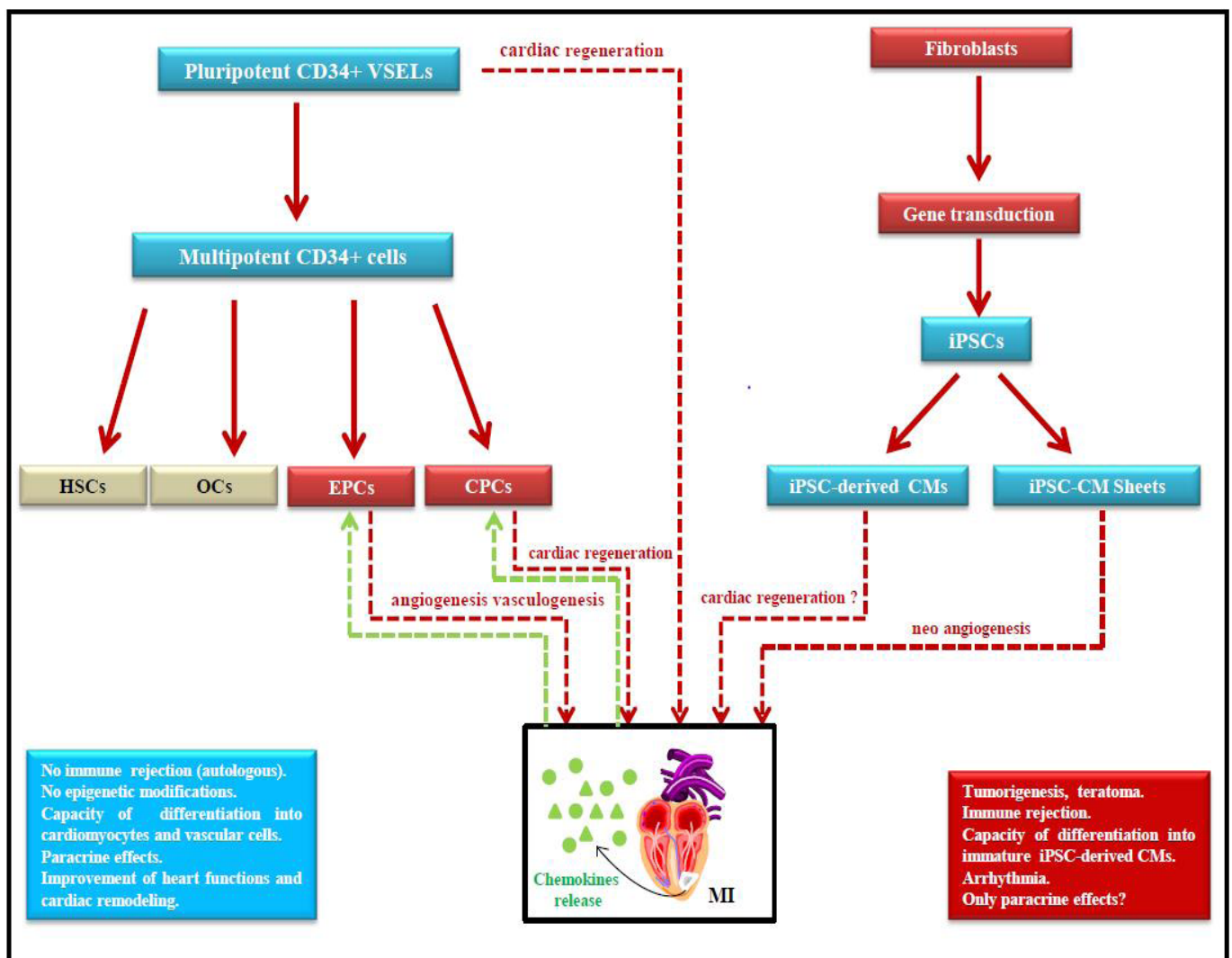

Figure 1: Regenerative medicine in ischemic heart disease. Advantages and limitations of CD34+ and iPSCs cell-based therapies in the treatments of myocardial infarctions. HSCs indicates hematopoietic stem cells; OCs, osteogenic cells; EPCS, endothelial progenitor cells; CPCs, cardiac progenitor cells; iPSCs, induced pluripotent stem cells; CMs, cardiomyocytes; MI, myocardial infarction

Conclusion

Besides ESCs, other cell types have been retained by different groups of researchers (i.e., CD34^{+}, VSELs and iPSCs) to determine the benefits and drawbacks related to their future development with a view to applications in cardiac regeneration (Figure 1). Purified, CD34^{+} stem cells taken from blood or bone marrow have displayed effectiveness in the treatment of blood diseases, but other unexpected therapeutic dimensions related to these cells have been identified in recent years. Despite the emergence of iPSCs and the hope they have aroused as an alternative to overcome the frustrating problems raised by ESCs, no clinical trial has been successfully achieved to date. The main hurdles to overcome are the risk of tumorigenesis, the development of life-threatening arrhythmias and immunogenicity. Moreover, engineering iPSCs is a lengthy and costly process. The choice of manufacturing surgically implantable cardiac sheets from iPSCs, with the only goal being to achieve a hypothetic paracrine effect promoting neo-angiogenesis, rather than cardiac tissue repair, is likely clinically and economically not very acceptable. The iPSCs should not be realistically employed in the clinic in the foreseeable future until significant progress in their clinical safety has been achieved. At the moment, they only serve as models to test *in vitro* drugs' toxic effects on cell survival, genetics, or differentiation, or as tools for developing experimental models to study genetic diseases. Even in such cases, data obtained using iPSCs must be cautiously evaluated because of their genomic instability and variability.

It is worth considering whether isolated and expanded VSELs could be safer and more efficient to improve heart function and cardiac remodeling when used in regenerative medicine. They represent a credible alternative to ESCs and iPSCs, as an easier-to-access source of purified pluripotent CD34^{+} stem cells for regenerative medicine, with no ethical issues, no epigenetic modifications and no undesirable side effects as risk of teratoma or cancer formation. Furthermore, CD34^{+} cells—consisting of about one fifth of VSELs—have already been demonstrated to be effective as a treatment for MI [24]. However, technical issues remain for VSELs, particularly the necessity to improve expansion-folds to achieve an optimal number of cells. Additional studies are therefore needed to prove the clinical potential of purified VSELs.

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