Non-resident stem cell populations in regenerative cardiac medicine

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Abstract. The adult heart displays a low proliferation capacity, compromising its function if exposed to distinct biological insults. Interestingly, the observation that an increasing number of cell types display an unpredicted cellular plasticity has opened new therapeutical avenues. In this review we will summarize the current knowledge of non-resident stem cells that can be putatively used for cardiac regeneration. At present, bone marrow stem cells have been extensively studied as a cellular source to heal the heart; however, their myocardial contribution is highly limited. Experimental studies have demonstrated that skeletal myoblasts can engraft into the heart, although, unfortunately, they lead to myocardial uncoupling. Embryonic stem cells can spontaneously generate cardiomyocytes that exhibit a variety of electrophysiological phenotypes. Several constrains should nonetheless be overcome before entering the clinical arena, such as the ability to direct and control the generation of cardiomyocytes into a single myocardial lineage.

Keywords. Cardiac regeneration, cell therapy, stem cells, embryonic stem cells, bone marrow stem cells.

Introduction

In the adult, the heart is an essential organ with low proliferation capacity since the majority of the myocardial cells become terminally differentiated soon after birth [1]. Such a low proliferative capacity results in a lack of early response if exposed to several types of insults, such as low oxygen supply (hypoxia) or blood insufficiency (ischemia) [2, 3]. Depending on the biological insult, the damage suffered by the heart can lead to sudden death, or in the best case, it can develop into compromised maladaptive situations such as hypertrophic and/or dilated cardiopathy [4]. In the long run these processes lead in many cases to a high incidence of undesired arrythmogenic events and/or sudden cardiac death [4]. Thus, the clinical scenario dictates the need to search for strategies which will combat heart disease specifically aiming to replace and/or regenerate damaged myocardial cells.

Over the last few years, we have been experiencing a biomedical revolution in the cardiovascular field with the advent of regenerative medicine [5, 6]. The observation that an increasing number of adult cell types display a high level of plasticity has opened up new therapeutical avenues [7–9]. Furthermore, the isolation and characterization of pluripotent human embryonic stem cells has increased these possibilities [10–12]. The initial experimental assays using these cell sources resulted in highly promising observations that have been pursued by recent clinical trials, generating a moderate optimism. Furthermore, the recent discovery that the adult heart possesses a pool of resident multipotent cells that are able to differentiate into distinct cardiovascular lineages is of great interest as a potential therapeutic target. The precise characteristics of these cellular subpopulations as well as therapeutical approaches using these cells are treated in detail in two review articles in this issue [13, 14].

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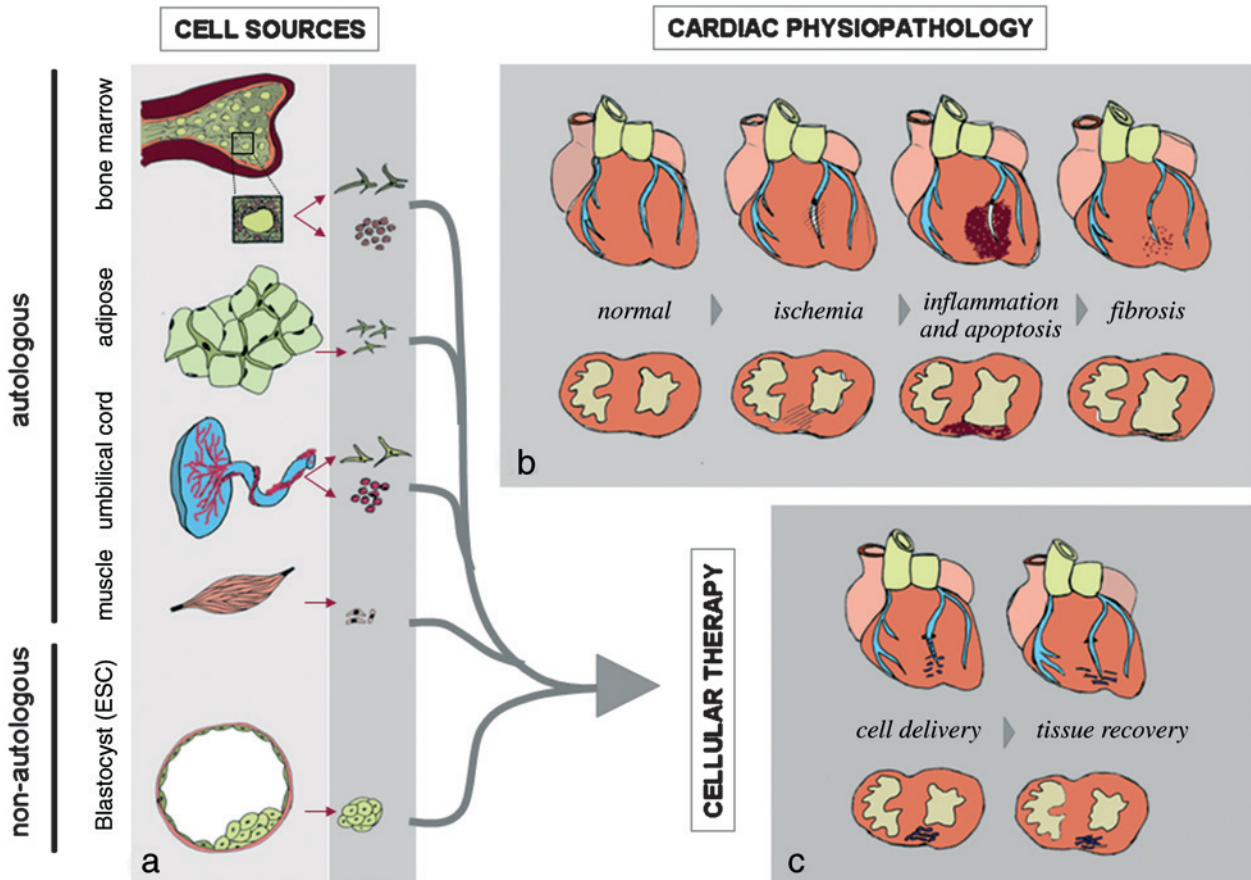
Figure 1. Schematic drawings illustrating the source of distinct cell populations used for cardiac regenerative medicine (a), the natural history of cardiac ischemic insult (b) and the cellular approaches (c) to healing the damaged heart.

At present, there are reasonable arguments for persuing new strategies for healing the damaged heart. The search for adequate cell populations to be used in regenerative cardiac medicine is being explored, and in several cases there is clear evidence that those cells can convert into cardiomyocytes [5, 15–17]. Ideally, if we aim to use a cell population to restore myocardial damage in the heart, these cells should be obtained from the same patient to be treated (autologous) therefore avoiding immunosuppressive therapy. Second, they should be easy to isolate and in relatively large quantities. And third the derivation of these cells to the cardiomyogenic lineage should be efficient and largely controlled to give rise to the expected/desired cardiomyocyte type, including robust functional and electromechanical coupling. To date, none of the cell sources that have been reported entirely fulfill these requirements but extensive efforts are being devoted to such studies, which should lead, with a reasonable chance of success, to an age of regenerative cardiac medicine.

In this review we will summarize current knowledge of non-resident autologous and non-autologous cell populations that have been reported to provide a source of cardiomyocytes (Fig. 1). Within the autologous cell populations we willl include bone marrow stem cells, adult skeletal myoblasts, adipose tissue stromal cells and umbilical cord stem cells, whereas for non-autologous cells we will illustrate the advances in using fetal/neonatal cardiomyocytes as well as embryonic stem cells. We will highlight the current state of the art regarding the cellular and molecular mechanisms of cardiomyocyte lineage determination and well as promising features and technical limitations of the different cell sources.

Autologous non-resident stem cells for cardiovascular medicine

The use of autologous stem cells for cardiovascular medicine has major advantages since the host will recognize the transplanted cells as its own. Inflammatory processes and rejection are thus avoided and the host will not require immunosuppressive therapy. Although this is indeed a major benefit, there are

nonetheless several limitations to be taken into account. At present, four non-resident stem cell sources have been reported to generate cardiomyocytes *in vitro* and/or *in vivo*, and thus can be considered as putative sources for autologous cell therapy: a) bone marrow stem cells (haematopoietic and mesenchymal lineages), b) skeletal myoblasts, c) adipose tissue stromal cells and d) umbilical cord stem cells.

Bone marrow stem cells

The bone marrow hosts two different stem cell types, haematopoietic and the stromal/ mesenchymal stem cells. Haematopoietic precursors give rise to the lymphoid and myeloid cell lineages [18], whereas stromal cells can differentiate into distinct mesodermal lineages such as osteoblasts, chondrocytes and skeletal myocytes [19].

The initial observations that haematopoietic stem cells (HSCs) from the bone marrow could form new myocytes in the ischemic mouse heart aroused great interest in the cardiovascular field [5]. These experimental observations led rapidly to phase I clinical trials that resulted in mild to moderate functional recovery [20–22]. However, at the same time, several other groups were reporting contradictory data regarding the rate of cardiomyocyte formation and engraftment into the heart from HSCs [23, 24], thus giving rise to a great deal of caution on the onset of further clinical trials. Recently, new evidence shows that HSCs do indeed contribute to the formation of the interstitial tissue in the adult unchallenged heart [25]. These contradictory data are presently halting the putative therapeutical usage of HSCs in ischemic heart diseases until confirming data are available.

Mesenchymal stem cells isolated from the bone marrow, and expanded in culture, have the potential to contribute to multiple cell lineages, including cardiomycytes [26, 27]. Cardiomyogenic differentiation has also been documented using a myocardial infarction model [28–30]. However, their therapeutical significance is unclear due to the scarce cellular incorporation rate [28–30].

An important line of debate concerns the cellular mechanisms by which bone marrow stem cells (BMSCs, haematopoietic and/or mesenchymal) contribute to the heart. At present, several experimental reports have provided compelling evidence that BMSCs engrafted into the myocardium of the normal adult heart do so by fusing with preexisting myocytes [31, 32] as also occurs in other tissues [33, 34], whereas no fusion seems to be involved in the generation of interstitial tissue [23]. However, this is far from clear since other reports equally attribute cardiomyocyte formation to differentiation and/or fusion events [7, 35]. A second issue which is more difficult to resolve

concerns how human BMSC engrafted into the ischemic heart may influence initial short-term functional recovery [20–22]. Experimental data using small animal models have demonstrated that engraftment of BMSCs into the normal and/or triggered heart is rare [27, 28]. Thus, how is it possible that hBMSCs elicit positive functional recovery if engraftment as new myocytes is rare and does not add significant myocardial mass? There could be several explanations for this that should be explored in detail: a) the functional recovery observed in human patients is simply due to the mechanism by which the cells are delivered into the coronary arteries in those patients and is not at all related to the cellular contribution of the BMSCs, b) the contribution of BMSCs to myocardium, although highly limited at the cellular level, might be effective for the formation of new vascular beds, c) the contribution of BMSCs is basically zero at the cellular level yet provides key soluble factors (growth factors and cytokines) that are able to promote and govern the recovery of damaged myocytes and/or the formation of new capillary vessels (Fig. 2). To date we only have partial answers to these questions.

We currently know that the contribution of BMSCs seems to be more than merely a technical catheter-based cell delivery system since recent phase I clinical trials suggest a significant functional recovery in patients treated with BMSCs compared with controls that underwent cathered-based placebo treatment [36]. However, the cellular contribution to the ischemic heart using these approaches in human patients seems very limited. Experimental studies using both normal adult hearts and experimentally challenged specimens also demonstrated that the number of cells that engraft the adult heart is basically insignificant both at the myocardial and the endothelial level [N.M. and D.F., unpublished data]. Thus it is more plausible to envisage that BMSCs are indeed providing/delivering key bioactive molecules that promote the ingrowth and/or survival of the challenged cardiac tissue. Along this line of argument, recent reports have observed that homing signals such as those elicited by the stromal-derived factor 1 (SDF-1) can help to provide cues for the BMSCs to nest into the heart [37].

In summary, BMSCs have been extensively studied as a cellular source of new myocytes for the damaged heart. The fact that these cells can be obtained in large quantities from patients who have suffered cardiac insults and the long-standing characterization of blood transfusion therapies constitute huge advantages for this approach to cardiovascular regenerative medicine. Many papers have provided evidence that several subpopulations of BMSCs can engraft into
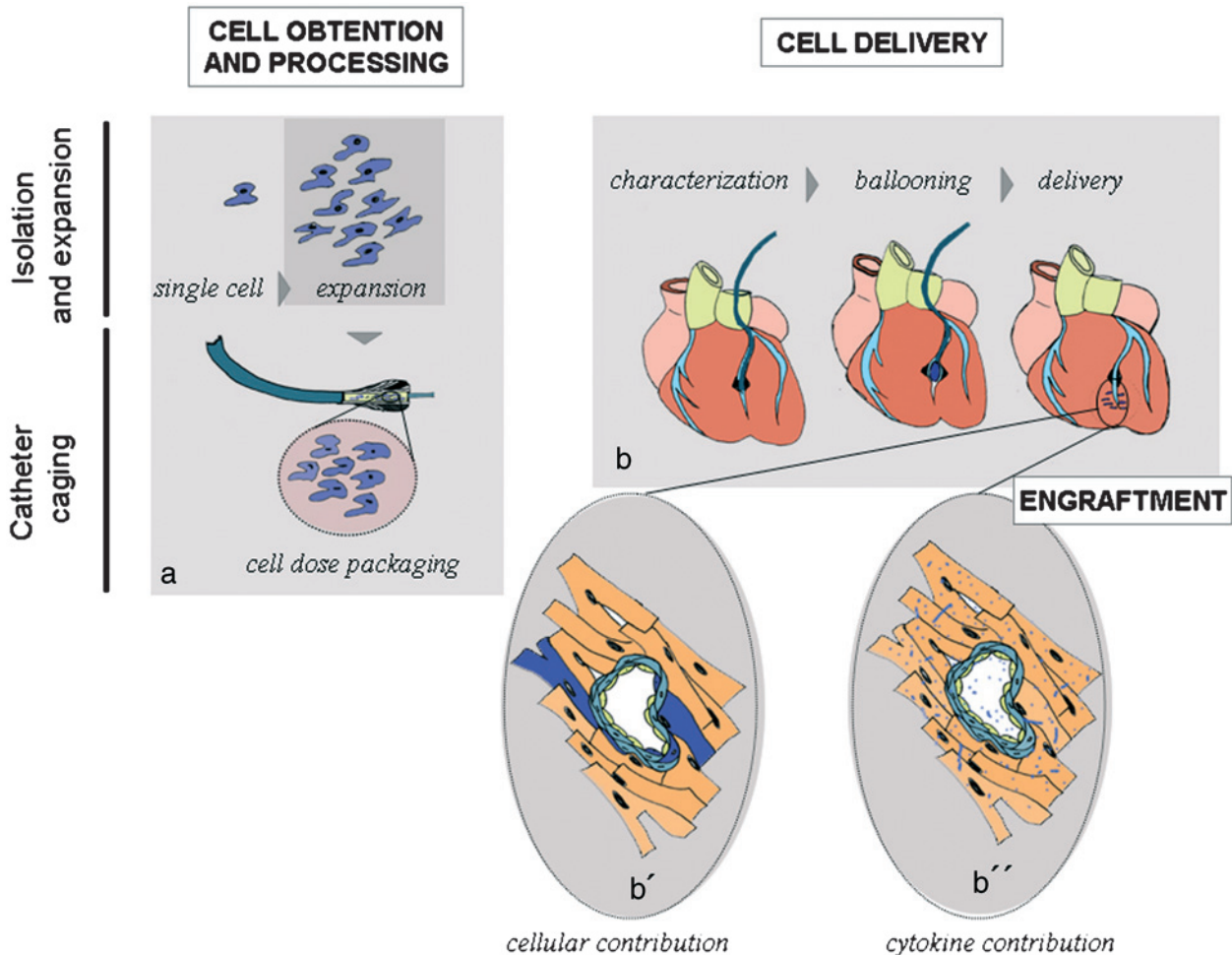
Figure 2. Schematic drawings illustrating the process of cardiac regenerative medicine starting from the isolation and characterization, in vitro expansion and catheter packaging of a cellular pool (a), to the *in vivo* delivery into a damaged heart (b). The contribution of this delivery can be either to increase the cellular component (B′) or to release growth factors/cytokines that might enhance the insulted cells ability to heal and recover (B″).

normal as well as experimentally challenged hearts. However, the net cellular contribution to the myocardium in both experimental and clinical settings remains controversial. A reconciling hypothesis would be that although the total cellular contribution to the damaged heart might be scarce, the contribution of growth factors and/or cytokines derived from transplanted BMSCs is highly beneficial. Thus, new paths should be taken to unravel these signalling pathways and their putative use as therapeutic tools.

Skeletal myoblasts

The use of skeletal myoblasts which are morphologically and functionally similar to cardiomyocytes has been pursued for almost a decade [38–40]. In fact, skeletal myoblasts can easily be obtained from the cardiovascularly damaged patient and can be cultured *in vitro* where they maintain native morphological and functional characteristics [41]. Furthermore, since

skeletal myoblasts and cardiomyocytes share many functional and electromechanical characteristics, there are evident advantages to this choice for cellular therapy of the heart [42]. Experimental studies have demonstrated that cultured skeletal myoblasts, if transferred to the normal adult heart, are able to engraft into the myocardium [42, 43]. However, when these skeletal myoblasts were grafted in significant amounts in a clinical setting, to cardiovascularly injured patients, cellular engraftment occurred adequately, but no functional coupling was obtained, leading to the generation of arrythmogenic foci [44, 45]. These observations suggest that skeletal myoblasts cannot be used in the clinical setting unless mechanisms to enhance electromechanical and functional coupling of the newly transplanted cells and the host cells can be achieved.

Thus, whereas skeletal myoblasts can be used in an autologous way and the lack of large initial material

can be overcome using *in vitro* culture expansion systems, the inability of these cells to provide functional coupling, resulting in undesirable arrythmogenic events, lends a difficult perspective to this cellular subpopulation for myocardial repair unless coupled with cellular and/or molecular strategies that overcome these barriers.

Adipose stromal stem cells

The advent of regenerative medicine has enhanced the search for novel sources of multipotential cells. In this search, it has recently been reported that stromal cells from adipose tissue, if cultured under specific cell culture conditions, are able to generate beating cardiomyocytes [15, 46]. Molecular analyses have documented that cardiomyocyte-specific molecular markers such as cardiac-enriched *Nkx2.5*, *Mef2c* and *Gata4* transcription factors as well as sarcomeric markers such as *Mhc* and *Mlc* isoforms are expressed in these cells, yielding to electrophysiological recordings similar to adult cardiomyocytes [15]. However, to date, we do not know whether these adipose stromal-derived cardiomyoyctes are capable of engrafting into the adult normal heart and if so, whether they will be correctly electromechanically coupled. The molecular hallmarks of these cells remain to be established, as does their abundance and distribution. These are therefore early days for this cellullar subpopulation in regenerative medicine since only the *in vitro* potential of this approach is guaranteed. A great deal of basic cellular and molecular studies is desirable before the adipose stromal stem cells can be used in clinically oriented therapeutic approaches.

Umbilical cord stem cells

The blood supply that circulates during fetal development includes a small proportion of stem cells that, if stored under relatively standard cryopreservation conditions, can be used even after 20 years for functional recovery of the haematopoietic lineage [47]. These observations led to the generation of public and private umbilical cord cell banks almost 40 years ago in the USA and more recently in several European countries [48]. The emergence of regenerative medicine as a new and highly promising discipline has generated much interest in the cellular and molecular properties of these cells as therapeutic tools [48]. However, it has also generated a great deal of debate about their plausible use as autologous or non-autologous delivery, which is beyond the scope of this review [48].

Several reports have indicated that umbilical cord stem cells can generate new endothelial vessels in vitro [49]. Recently, evidence of myocardial cells generated from umbilical cord stem cells was also reported [17], although any putative therapeutical usage remains to be explored. Similarly to the previously reported adipose stromal-derived cardiomyogenesis, these are early days for the possible use of umbilical cord stem cells in cardiac regenerative medicine.

Non-autologous, non-resident stem cells for cardiovascular medicine

The limitations outlined in the previous paragraphs as well as the great potential that some non-autologous cell sources have for regenerative medicine has led to a situation where non-autologous cell sources are progressively becoming better candidates for cardiac regeneration. At the same time, new strategies to overcome and/or bypass the issue of tissue rejection are becoming available, such as the future generation of engineered stem cells by somatic nuclear transfer [50]. In this section we summarize the potential contribution of two non-autologous cell sources for cardiac healing: a) fetal cardiomyoblasts and b) embryonic stem cells. Whereas the first approach explores the use of homologous cell populations to heal the damaged heart, immunosuppressant therapy is imperative. In the use of embryonic stem cells, however, genetic manipulation might, in the near future, allow adaptation of cells to the host immunological system.

Fetal/neonatal cardiomyoblasts

Over many years, the unique alternative to serious cardiac damage has been heart transplantation. The lack of sufficient immunologically compatible and temporally appropriate donors has launched the search for innovative alternatives. Among them, the use of fetal cardiomyoblasts obtained from programmed abortions was explored [51]. The use of fetal cardiomyoblasts has produced some promising results in experimental settings [51]. However, the number of clinical experiences, although promising, is rather low due to the complexity of the system [52]. Furthermore, the possibilities of scaling up this approach into a clinically relevant situation are rather unlikely because of the expected mismatch between available fetal cardiomyocyte resources from consented programmed abortions and the greater number of putative recipients.

A more promising landscape can be obtained using a combined approach of selecting neonatal cardiomyoblasts and using compatible biomaterials. Such approaches are being currently explored in experimental models as heart-assisted devices, using engineered tissue grafts that provide very encouraging results [53, 54]. However, as pointed out by the authors [52],

scaling up this approach to target the damaged human heart encompasses several challenges that should be stepwise resolved.

Embryonic stem cells

Embryonic stem cells (ESC) are highly plastic cells that can spontaneously differentiate into distinct cell lineages [55, 56]. Mouse embryonic stem cells have been routinely cultured *in vitro* for over 2 decades [56–58]. It is currently well documented that ESC, if cultured as embryonic body aggregates, lead to the formation of beating cardiomyocytes. Such ESC-derived cardiomyogenesis displays a similar gene expression profile to the endogenous cardiomyogenic lineage [59]. More interestingly, ESC-derived cardiomyocytes elicit a variety of electrophysiological phenotypes, suggesting that different cardiomyocyte types are generated [57, 58], similar to *in vivo* cardiogenesis. These properties, as well as the possibility of scaling up ESC cultures and engineering them by somatic nuclear cell transfer, provides the basis for launching ESC as a highly competitive and promising source of cardiomyocytes for regenerative medicine. However, there are several constraints that should be taken into account before moving into the clinical arena. First of all, while we can generate cardiomyocytes from ES cultures, this process is uncontrolled and remains exclusively spontaneous. Cytokine treatment can increase the number of beating areas [60], although it remains to be explored whether all the cardiomyocytes become confined to single or distinct myocardial lineages. Second, to date, most reported experiments are based on *in vitro* culture conditions, whereas scarce data *in vivo* are available [61, 62]. Ideally, we would aim to isolate beating areas and transplant them into the adult heart to observe whether they are able to engraft into cardiac muscle and to be electromechanically coupled. Two critical questions are a) whether engraftment of ESC-derived cardiomyocytes leads to oncogenic processes and b) whether ES-derived cardiomyocytes, after engraftment, generate arrythmogenic foci. We can foresee that even if a few undifferentiated cells are transplanted into a host heart, these cells may eventually lead to the formation of teratomas. Even if this does not occur during the lifespan of a mouse ($2-2^{1/2}$ years), a great deal of caution is warranted, considering the longer human lifespan. Finally, if ESC are innocuous regarding their oncogenic potential, and are properly engrafted and electromechanically coupled, we will need to have a homogeneous cardiomyocyte lineage cell population, i.e. ventricular cardiomyocytes that should not lead to arrythmogenic events. At present, we lack specific knowledge about the molecular cues that govern the commitment of mesodermal cells to the cardiomyogenic lineage, although cardiac developmental biology is progressively increasing our understanding [63, 64]. Further insights into the basic developmental processes that govern myocardial lineage commitment are crucial for the development of strategies to control and eventually transplant ESC-derived cardiomyocytes into the damaged heart. Furthermore, these findings will need to be confirmed using suitable human ESC.

Human ESC have only been obtained over the last few years [65], and their experimental usage remains highly controversial with regard to ethical and moral concepts, which is beyond the scope of this review. hESC are more difficult to isolate than mouse ESC, including ethical and legal constraints that vary between different countries. At present, experimental data using hESC demonstrate that these cells display a more restricted pluripotency, as compared with mouse ESC, becoming committed to the cardiomyogenic lineage only if triggered exogenously [66–68]. To date, little information is available about whether hESC undergo a similar 'developmental' program to that previously reported for mouse ESC, or whether they generate only a single cardiomyocyte lineage [69]. Over the coming years, a large number of initiatives will be undertaken to provide a suitable number of ESC lines to experimentally design therapeutic strategies as well as to provide basic cellular and molecular mechanisms to master the cardiomyogenic cell commitment process before moving into clinical practice. Beside these technical considerations, which will most likely be addressed in the relatively near future, there is a major drawback to using hESC, and that is the immunological distress that they might cause in the host patient due to tissue histocompatibility mismatch. Immunosuppressant therapy might not always be applicable and/or recommended. Recently, hope has been raised by advances in somatic nuclear cell transfer [70], although much research remains to be done before entering the clinical arena.

Perspectives

Over the last few years, hopes have been raised that regenerative medicine will soon cure several physiopathological conditions, including cardiac pathologies such as ischemia, eliciting great social demand. In the short term, scientific achievements are too premature to be significant in clinical practice; however we are confident that in the long run, regenerative medicine will have an important impact. There is a long way to go yet before real and applicable cardiovascular regenerative medicine is widely used and brings high-impact benefits to patients with heart disease.

The routes to be explored in the near future should mainly be fundamental. Firstly, we should learn whether the ischemic heart requires new myocytes or new vasculature, or whether, on the contrary, a supply with specific growth factor/cytokines would be even more beneficial. Therapeutic approaches may be very different depending on the most effective molecular and cellular mechanisms.

Second, if cell transplantation is required, we must obtain insights about how many cells engraft in the damaged human heart and which cell types are produced, based on the cellular origin of the transplanted cell pool. At the same time, we should develop adequate experimental animal models (large animal models) that can both molecularly and functionally mimic the human situation. It is clear that the early experimental data on small experimental animal models can give us very valuable information regarding the molecular and cellular mechanisms of regenerative medicine, but they are highly limited for issues of size and function. A key question that remains elusive is to learn which cell type and subpopulation should be used for transplantation. Efforts to unify concepts in this respect are required and should lead to significant progress in the near future.

Third, only if the previous requirements have been met, should we embark on designing new clinical trials to formulate answers regarding as which cells, how many and when and where and how should be delivered into an ischemic patient, which will lead in turn to new and more complex clinical trials.

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