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CONCISE REVIEW

Stem cell self-renewal: lessons from bone marrow, gut and iPS toward clinical applications

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The hematopoietic stem cell (HSC) is the prototype organ-regenerating stem cell (SC), and by far the most studied type of SC in the body. Currently, HSC-based therapy is the only routinely used SC therapy; however, advances in the field of embryonic SCs and induced pluripotent SCs may change this situation. Interest into *in vitro* generation of HSCs, including signals for HSC expansion and differentiation from these more primitive SCs, as well as advances in other organ-specific SCs, in particular the intestine, provide promising new applications for SC therapies. Here, we review the basic principles of different SC systems, and on the basis of the experience with HSC-based SC therapy, provide recommendations for clinical application of emerging SC technologies.
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Introduction

Stem cells (SCs) can be classified as embryonic or adult, depending on their tissue of origin. Adult SCs sustain an established collection of mature cells over the lifetime of the organism. Proliferation of SCs without loss of key characteristics such as self-renewal and multilineage differentiation potential is an important topic in SC research.[1] The hematopoietic SC (HSC) is the prototype organ-regenerating SC, and by far the most studied type of SC in the body.[2] HSCs give rise to all types of peripheral blood cells including lymphocytes and myeloid cells. In adult vertebrates, hematopoiesis normally occurs in the bone marrow (BM) and requires a unique microenvironment, referred to as the SC niche.[3] In the niche, a three-dimensional network is created by stromal tissue. Extracellular matrix components and hematopoietic growth factors promote SC maintenance and regulate self-renewal and differentiation. Similar niches for adult SCs exist in the gastrointestinal tract, muscle and skin.[4–12]

With the recent focus on embryonic SCs (ESCs) and induced pluripotent SCs (iPSCs), interest into *in vitro* generation of HSCs, including signals for HSC expansion and differentiation from these more primitive SCs, has seen a tremendous boost.[13–15] As HSC-based therapy is the only type of SC therapy routinely used, much knowledge obtained from the clinical application of HSCs and from genetic modification of HSCs for gene therapy applications can be used to introduce other types of adult SC therapies, and perhaps in the future extend this to include ESCs and iPSCs.

At the third Wadden symposium on the Dutch Island of Texel (27–30 June 2010), SC biologists and clinicians from various backgrounds gathered to discuss mechanisms for self-renewal and obstacles toward clinical application of SCs for regenerative medicine. The review here is, in part, based on discussions held among the authors, who were participating in this conference.

iPSCs: generation of ESC-like SCs from somatic cells

Pluripotent cells have the ability to differentiate into any of the hundreds of different cell types of the body; ESCs, derived from early (preimplantation) embryos, are generally considered the prototype pluripotent SC, but germ cells and the epiblast of postimplantation embryos can also give rise to pluripotent cell lines in culture. Multipotent SCs, such as the HSCs, have a more limited differentiation potential and are able to form only a few different mature cell types.

ESCs are also capable of potentially unlimited self-renewal while maintaining the potential to differentiate into derivatives of all three germ layers (ecto-, endo- and mesoderm). Mouse ESCs were isolated over 30 years ago and paved the way for the isolation of human ESCs in 1998. Much of the anticipated clinical potential surrounding human ESCs is an extrapolation from pioneering experiments in the mouse system.

In 2006, Yamanaka and colleagues[16] at the Kyoto University reported that the introduction of genes encoding four important SC transcription factors (Oct4, Sox2, KLF4 and c-Myc) into adult mouse cells by retroviral transduction resulted in reprogramming them into cells with ESC-like properties. These

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reprogrammed cells were referred to as 'iPSC,' for induced pluripotent stem cells. In 2007, the Yamanaka group[17] and the laboratory of Thomson[18] (Wisconsin) described the successful genetic reprogramming of human adult cells into human iPSCs. Such cells hold an enormous potential for disease modeling, drug testing and eventually transplantation for regenerative purposes.[19] Thus, ESCs and iPSCs share many similarities and are highly similar in gene expression profile and functional properties, despite their obvious different origins, as iPSC are by definition man made.

The expected impact of human ESCs and iPSCs in medicine includes:

- Cell transplantation therapy that derives from the ability of ESCs to differentiate terminally into various tissue cell types, such as neurons, cardiac and vascular cells or blood cells.
- Insights into genetic disease when the ESCs are derived from embryos identified in prenatal genetic diagnosis as bearing specific genetic mutations. iPSCs can be generated from cells taken from babies or adults of all ages with full medical records and suffering from virtually any genetic disease, whether simple or complex. With more than 5000 known genetic diseases, it would appear that considerable information might be gained from using iPSCs to study them including pathophysiology of disease, discovery of new prognostic biomarkers and a continuous supply of afflicted cell types for drug screening and discovery.
- Following targeted genetic modification of these cells, genetic disease models can be created in the laboratory. Targeted genetic modification of patient-derived iPSCs could result in genetically repaired cells suitable for (autologous) cell therapy, as already successfully applied to HSCs in gene therapy for certain primary immunodeficiencies.

Particularly in the field of cardiac regeneration and the study of cardiac disease, significant progress has been made in deriving and characterizing cardiomyocytes from ESCs and iPSCs.[20] Although the earliest papers on the derivation of cardiomyocytes from hESCs reported that at most 1% of the cells in a differentiated cell culture were actually cardiomyocytes, efficiencies under defined conditions can now reach up to ~30%.[21] When combined with genetic or marker-based selection techniques, it is now possible to obtain cultures entirely composed of cardiomyocytes. Although the problems associated with transplantation of these cells to the heart for heart repair, for example, after a myocardial infarction, remain manifold and cell therapy for the heart is very much a far future application,[21] the ability to generate these pure populations of cardiomyocytes are actually opening up equally exciting areas of different kinds of research in safety pharmacology and drug discovery.[22,23] Likewise, vascular endothelial cells derived from ESCs can provide vital clues in drug target identification and action.[24]

Progress in the generation of iPSCs is rapid. Although initially γ-retroviral or lentiviral vectors have been used, later developments include lentiviral vectors that can be excised (integration-free), other types of transposons, synthetic mRNAs, protein delivery or pharmaceutical approaches, all aimed at inducing the pluripotent stage (reviewed in Stadtfeld and Hochedlinger[25]).

The signals responsible for reprogramming iPSCs and the self-renewal pathways of ESCs are closely related but not identical. Systems biology approaches have identified several important pluripotency pathways including LIF/gp130/STAT3, BMP4/STAT5, OCT4/Sox2, Nanog/Tcf3 and ERK and GSK3β. The latter two differentiation signals need to be inhibited to preserve pluripotency.[26–29] In such approaches, gene expression profiling, miRNA profiling and measurements of alternative spliced RNAs as well as mature proteins are all integrated into complex computational biology approaches. Such experiments may also shed light on the self-renewal programs of adult SCs, which remain largely unknown and have to be different from those of ESCs. Moreover, for clinical use these embryonic or ESC-like engineered cells have been shown, in some cases, to differentiate into mature functioning tissue cells,[19,30–32] although many cell types derived from SCs remain immature. The benefits of pluripotency should be evaluated against the risks of the latter, for example, acquisition of genomic instability on prolonged culture,[33] uncontrolled growth by undifferentiated rogue cells present in transplanted cell populations, ectopic tissue growth and malignancies such as teratomas.

Of interest, Ratajczak and coworkers[34] identified a population of SCs in adult BM that seemed to be similar to early ESCs in terms of morphology and marker expression and termed these cells very small embryonic-like SCs. These cells are present in many adult tissues, including BM, and could be harvested from cord blood. Very small embryonic-like SCs could be a useful source of SC for regenerative medicine applications.[34–37]

Adut SCs: gut versus blood

Adult tissues with a high turnover rate, such as blood, skin and intestine, are maintained by tissue-specific SCs. Tissue SCs themselves rarely divide, but the so-called transient amplifying progenitor cells do, although intestinal SCs are believed to divide rapidly, much more so than blood SCs. There is a striking resemblance between intestine and blood SCs and differentiation pathways. In both systems, SCs are located in a specific microenvironment referred to as niches. Here, SCs self-renew and differentiate into rapidly dividing daughter cells (transient amplifying cells or multilineage progenitors) that can give rise to various different types of cells (for example, absorptive cells, goblet cells, paneth cells and enteroendocrine cells in the gut, red cells, platelets and the various leukocyte lineages in peripheral blood; see Figure 1). However, gut SCs divide more rapidly than HSCs.

Of interest, many of the pathways suggested for hematopoietic differentiation from ESCs and iPSCs are also defined in development and differentiation of SCs in the gut, where several morphogenic pathways, such as the Wnt, Hedgehog (Hh) and tumor growth factor-β family, have a key role in maintaining a homeostatic equilibrium between SCs and their differentiated progeny. Intestinal epithelial SCs reside in crypts, where their fate and proliferation depends on Wnt and Notch signaling.[38] Differentiated cells secrete Indian Hh and signal to the mesenchyme to induce signals such as bone morphogenetic protein 4 and activins that negatively regulate fate and proliferation of intestinal SCs, thus maintaining a balance between SCs and differentiated cells.[39,40] Elegant work from the Clevers' laboratory has shown that intestinal SCs can be isolated using Lgr5 as a marker and can generate all intestinal epithelial lineages in defined medium in the absence of a niche,[41] although the transplantability of such cells is not yet clear. The epithelium thus grown *in vitro* shows a remarkable growth rate and is characterized by continuous crypt expansion by elongation and budding, a process termed crypt fissioning. The rate of crypt fissioning is very low in the normal intestine and occurs mainly during intestinal damage and repair (it is a hallmark of the pathology in ulcerative colitis) or during the formation of intestinal adenomas. Thus, perhaps the main

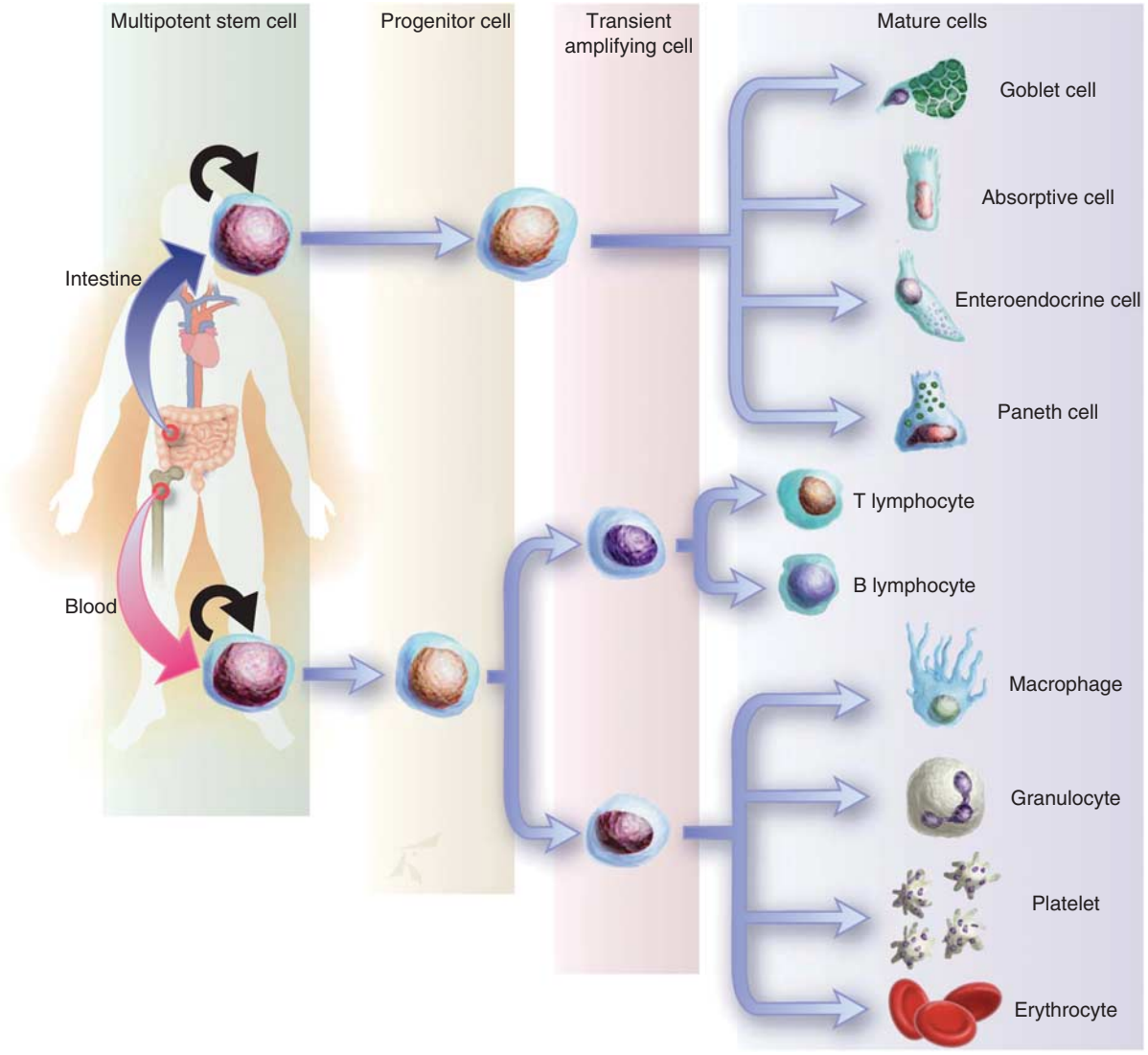

Figure 1 Comparison of intestinal and hematopoietic stem cells and their progeny. In both SC systems, self-renewing stem cells give rise to multiple cell lineages of mature cells. In between SCs and mature cells are populations of rapidly dividing progenitor cells. Intestinal stem cells make use of their progeny as niche cells. Therefore, the anatomical restriction of the crypt allows them to form niche cells by themselves. This seems to be a strong contrast to HSCs, which require the mesenchymal and osteoblastic niche cells. Much more complex differentiation models of hematopoiesis have been described; however, the figure was designed to show similarities rather than point out differences.

function of the mesenchyme (or niche if there is such a thing in the intestine) is to restrict the activity of the epithelial SCs and prevent excessive crypt expansion. Perhaps, extrapolation of gut SC data will prove favorable to further advance in the field of eventual HSC generation and expansion.

Generation of HSC

Despite the vast experience with HSCs, many issues on the biology of HSCs remain unresolved. So far, it has been not possible to expand HSCs from adult sources without loosing self-renewal properties. As an alternative, the generation (and expansion) of HSCs from ESCs or from reprogramming of adult cell-derived iPSCs has not yet been accomplished either. On the other hand, iPSCs can be efficiently generated from the most specialized peripheral blood cell, the T lymphocyte.[42,43] However, HSCs are considered as the most useful cells for transplantation purposes and for regenerative medicine, compared with other blood cells that have been derived at low frequencies from iPSCs or ESCs. Clues on how to derive true HSCs from iPSCs or hESCs, with the ability to repopulate the BM, may come from studies on the ontogeny of HSCs.

In recent years, much progress has been made in the understanding of the development of HSCs in the embryo. The first wave of hematopoiesis occurs in the murine extraembryonic yolk sac between embryonic days 7 and 8. This transient hematopoietic system is mainly oriented to red blood cell production.[44] After the first wave of primitive erythropoiesis, adult HSCs, functionally defined by their capacity to confer complete, long-term and multilineage repopulation of the hematopoietic system of adult recipient mice, are first generated in the aorta–gonads–mesonephros region and can be detected at the beginning of embryonic day 10.5 in the mouse.[45,46] These HSCs are located in the ventral region of the dorsal aorta, in association with the endothelial wall.[47] On the basis of this

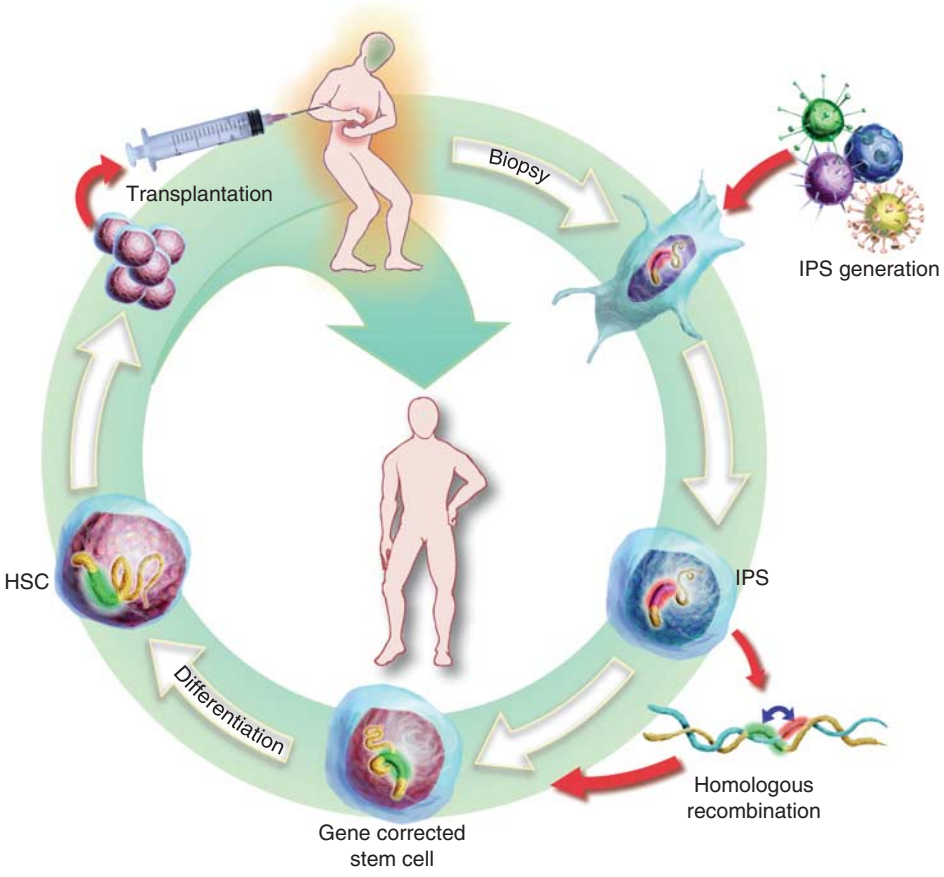

Figure 2 Use of IPS cells for correction of genetic diseases by gene-corrected stem cells. An alternative approach to viral-based gene correction is using patient-specific iPSCs and homologous recombination, followed by forced differentiation into HSCs. Generation of true, transplantable HSCs is still difficult to accomplish.

close proximity, it has been proposed that instead of a hemangioblast with both hematopoietic and endothelial potential, a 'hemogenic endothelium' lining the ventral aorta, from which the HSC emerge, could be the precursor of definitive HSCs.[48–50] This transdifferentiation of aortic endothelial cells to HSCs was recently shown in the mouse embryo by real-time confocal imaging.[48] Signaling pathways that may be important for development of HSCs include Wnt, Notch and bone morphogenetic protein signaling and transcription factors RUNX1(AML1), SIL/TAL and GATA.[51] Manipulation of these pathways may help in instructing an HSC fate on iPSCs. Such an approach, using patient-specific iPSCs and homologous recombination, followed by forced differentiation into HSCs, constitutes an attractive method for gene correction of inherited disease, avoiding the use of semi-randomly integrating vectors with potential genotoxicity (see Figure 2).

Genetic modification of HSCs

SCs can be genetically modified to overexpress genes of interest. This has been most extensively carried out with HSCs and has been successfully applied to cure several types of severe combined immunodeficiencies (SCID),[52–55] a collection of clinically severe diseases in which affected children miss adaptive immunity because of the lack of T lymphocytes and sometimes because of lack of B and natural killer cells. For SCID, underlying mutations in the common γ-chain (X-SCID), which forms the common component of receptors for interleukin 2 (IL2), IL4, IL7, IL9, IL15 and for SCID with defective adenosine deaminase, successful clinical trials have been undertaken in Paris (X-SCID), London (X-SCID) and Milan (ADA-SCID) (reviewed in Fischer *et al*.[56]). The trials for SCID-X1 have shown the clinical feasibility of introducing a therapeutic gene into autologous $CD34^+$ HSCs. Both SCID-X1 trials have been highly successful, showing long-lasting restoration of immunity.[57,58] The deficiency was restored and lymphocyte development was no longer blocked. However, the development of leukemia has appeared as a severe adverse effect. In all five cases ($n=4$ in the Paris, $n=1$ in the London trial), T-cell acute lymphoblastic leukemia occurred as a direct consequence of insertional mutagenesis by the retroviral vector used to deliver the therapeutic gene.[59–61] It is hoped that the development of novel vectors, especially those in which the viral promoter/enhancer sequences have been rendered inactive (self-inactivating vectors), will significantly reduce the incidence of insertional mutagenesis. This will likely promote the safety and thus further clinical development of cells that are genetically modified.[62–65]

The SC niche

The existence of a specialized niche or microenvironment that promotes maintenance of SCs was initially proposed in 1978 by Schofield *et al*.[66] Already by this time it was suggested that SCs are seen in association with other fixed-tissue cells that prevent

SC differentiation and ensure its continuous proliferation. However, only recently advances have been made to define their exact location as well as the molecular mechanisms by which they regulate HSCs. It is now clear that other types of SCs, for instance in colon, skin or mammary gland, also reside in niches. An interesting new prospect is that regulated competition between SCs occurs in the niches in which only the most fit SCs survive and contribute to the pool of differentiated progeny.

Within the niche, there is a unique molecular crosstalk between SCs and niche-constituent cells that normally maintains quiescence of SCs, but can mediate rapid activation of SCs in response to specific stimuli such as injury. One of the key functions of niches in adult animals is maintenance of quiescence, and self-renewal is progressively lost on proliferation. The signals underlying these properties are complex and include tumor growth factor-β and Wnt signals.[2,67–69] For instance, mice overexpressing the Wnt inhibitor Wif1 in niche osteoblasts show increased extra-medullary hematopoiesis and HSCs proliferate at the expense of self-renewal.

Another important niche signal is provided by the Hh pathway. The Hh signaling pathway in mammals consists of three closely related ligands, namely, Sonic Hh, Indian Hh and Desert Hh, that can each bind to the transmembrane protein Patched (Ptch). On ligand binding, Ptch inhibition of the positive effector Smoothened is released and signaling is transduced to the Gli transcription factors. Although a role for Hh in hematopoiesis has been controversial, recent work using Gli-deficient HSCs indicates that Hh regulates HSC and myeloid cell proliferation and differentiation.[70] In addition to Hh and Wnt signals, other niche signals, such as Notch signals, have recently been shown to be useful for *ex vivo* expansion of umbilical cord blood human HSCs.[71,72]

Recently, Frenette and coworkers[73] showed that mesenchymal SCs (MSCs), identified using Nestin expression, constitute an essential HSC niche component. Nestin$^+$ MSCs contain all the BM fibroblastic colony-forming units. Nestin$^+$ MSCs are spatially associated with HSCs and adrenergic nerve fibers, and highly express HSC maintenance genes in a manner regulated by sympathetic input. Conditional depletion of Nestin$^+$ cells compromises HSC homing and maintenance in the BM. Thus, two different types of adult SCs, HSCs and MSCs, are closely associated in the BM SC niche.[73]

The design of cell therapies should thus acknowledge the important interactions that these cells can have with the microenvironment they home to *in vivo*. These interactions will strongly influence SC behavior, for example, by regulating their differentiation and proliferation, and thus be determinant for the therapeutic efficacy and safety of clinical trials.

Toward clinical applications

HSCs have a long history of clinical use to treat a variety of malignant and non-malignant diseases of the hematopoietic system. In addition, the immunomodulatory properties of MSCs have also been used successfully for clinical applications, most notably in the prevention of severe graft-versus-host disease.[74]

Recently, MSC therapy has been successfully introduced clinically for a variety of indications. MSCs may be applied for their regenerative potential, that is, their ability to differentiate into bone and cartilage. Perhaps the most promising clinical application, however, might relate to their ability to modulate auto- and allo-immune responses. Encouraging results have been reported for the treatment of graft-versus-host disease following allogeneic SC transplantation and the treatment of *fistels* associated with Crohn's disease.[74] Sixty to eighty percent of patients with refractory acute graft-versus-host disease have responded to MSC therapy, although other effects have been moderate or non-homogeneous, or remain to be elucidated.

Further clinical application of both adult and ESCs or iPSCs, or otherwise genetically modified versions of MSCs and HSCs require a number of critical steps:

(1) Use disease-specific animal models to first test mechanisms of efficacy and also possible toxicity. Animal experiments will always be difficult to extrapolate to humans because of intrinsic differences in physiology and pathology.
(2) Test efficacy and safety using human cells in certain model systems *in vitro* and *in vivo*, such as immunodeficient mice. For gene therapy, gene-corrected patient cells are preferred as target cells in such models. Nevertheless, even the use of human cells in immunodeficient mice has limitations, as a model for human cells in humans.
(3) As much as possible standardize protocols and scale-up production of these cells, allowing testing of safety and efficacy as a pharmaceutical product, taking into account that these often autologous and custom-made therapeutic cells will differ from donor to donor and also between patients and healthy individuals.
(4) Conduct proper clinical trails with clearly defined end points, starting with the usual phase I and II studies with rigorous comparison of responding and non-responding patients (both in product qualities and in patient parameters before and after therapy) and eventually aiming for randomized clinical trials as a final goal.

The earliest methods for the induction of iPSCs relied on the use of viral vectors, which are associated with risks of insertional mutagenesis and transgene reactivation. These are still the most robust technologies that are being used in the multiple core production facilities established in many universities worldwide. However, numerous alternative methods for inducing pluripotency, which do not use gene insertion or effectively excise the reprogramming cassettes, have been reported, which should make it possible to bypass some of the safety concerns. However, others will still remain, such as the long-term karyotypic stability, appropriate localization of SC in the organs and potential for cancerous transformation.

Another concern with future SC therapies is that trials are inevitably costly, while the classical randomized clinical trial form, because of the thousands of permutations possible in isolation of cells, their manipulation, administration, patient categories and so on, represent an additional hurdle. Development of these therapies should therefore be considered within the consortia, in connection to major/large clinical pharmaceutical companies, acknowledging that these custom-made ('not off the shelf') products are a challenge to develop commercially or extensive government/not for profit support will be needed for such trials to proceed and for the eventual official (Food and Drug Administration, European Medicines Agency) registration of such therapies.

There are a number of other issues that we would like to mention here that affect the advance toward the clinical and SC therapies in chronic or acute disease. (Table 1: Recommendations). These include:

First, the hype associated with the field of SC therapy as a whole, in part resulting from sensational reporting by the lay press, but also in part resulting from some scientists seeking the non-scientific media to report small steps as 'breakthroughs'. Related to this are the numerous claims from *mala fide* SC clinics ('stem cell cowboys', 'stem cell tourism') offering (for

Table 1 Recommendations for knowledge dissemination and clinical application of experimental stem cell therapies

1 Avoid stem cell hypes. Claims should be based on solid scientific facts and clinical findings
2 Define standardized protocols for derivation, isolation, expansion, potential genetic modification and differentiation of stem cells. Rigorously established procedures that are reproducible on clearly defined cell types are essential
3 Free exchange of reagents and cells at an international level
4 Improve preclinical assays and biomarkers to assess potency and potential toxicity of stem cell products
5 Be restrictive in the use of patents. It is good to protect very specific intellectual property, but broad claims or not pursuing patents may hamper progress

instance on the Internet) SC treatments with claims of therapy not based on any scientific proof of efficacy. This further underscores the need for scientists to conduct appropriate experiments, especially in these highly experimental therapeutic settings. Making the distinction between 'experimental treatment' and 'unproven therapy' very clear for patients and their families is an essential responsibility of every physician. Moreover, scientists should clarify and provide realistic time lines for future therapies, when possible, to their supporters and sponsors, taking into account the fact that it will take more time than one might think to enter clinical trials (for example, it took 20 years after their discovery for monoclonal antibodies to have any clinical applications), and some diseases (for example, Alzheimer, multiple sclerosis and so on) may never be treatable by SCs. Scientists and physicians in the field should not be tempted to capitalize on the present hype and even more importantly on the benevolence of society and patient support groups. This will eventually reflect poorly on the field as a whole, as unmet promise can lead to pressure on first inhuman studies that could lead to serious adverse events, from worsening of the condition to fatalities through the 'rush into the clinic'.

Second, standardized protocols are highly important. Many different types of adult SCs have been reported, sometimes derived from a single laboratory, without independent confirmation by other laboratories. Standardized protocols for isolation and propagation of the cells, used with strict molecular and phenotypic definition of such cells, represent a critical issue.

Third, it follows that free exchange of reagents and cells is required. This should be done at an international level, preferentially in the context of international collaborations, such as in the EU framework programs. Exchange of scientists between different laboratories will also help.

Fourth, novel preclinical assays and biomarkers need to be developed to better predict the potency and potential toxicity of SC products.

Fifth, following the standardized international protocols, guidelines for clinical practices should preferentially also be developed. Thus, international harmonization of guidelines for the SC therapeutics is needed in the European Union, United States, Asia and Australia.

Finally, the (mis)use of patents is of concern. Although protection of intellectual property is important for attracting venture capital and commercialization of ideas, it may also hamper progress by discouraging new initiatives or in the worst case 'shelving' of useful methods and products by companies.

In summary, the clinical use of HSCs and MSCs has taught a great deal about how to progress with the use of these types of SCs beyond their traditional applications and set the stage for clinical use of other types of adult SCs and also of ESCs, very small embryonic-like cellss and iPSCs. The *in vitro* generation of HSCs, including signals for HSC expansion and differentiation from these more primitive SCs, as well as advances in other organ-specific SCs, in particular the intestine, provide promising new applications for SC therapies, including applications traditionally covered by hematological and immunological applications. The recent successful use of adult eye SCs for vision disorders is a good example of how solid basic experimentation followed by translational and clinical science application can successfully lead to novel therapies, while avoiding the hype of overambitious claims.

Conflict of interest

The authors declare no conflict of interest.

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