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STANDARDS, POLICIES, PROTOCOLS, AND REGULATIONS FOR CELL-BASED THERAPIES

Concise Review: Workshop Review: Understanding and Assessing the Risks of Stem Cell-Based Therapies

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ABSTRACT

The field of stem cell therapeutics is moving ever closer to widespread application in the clinic. However, despite the undoubted potential held by these therapies, the balance between risk and benefit remains difficult to predict. As in any new field, a lack of previous application in man and gaps in the underlying science mean that regulators and investigators continue to look for a balance between minimizing potential risk and ensuring therapies are not needlessly kept from patients. Here, we attempt to identify the important safety issues, assessing the current advances in scientific knowledge and how they may translate to clinical therapeutic strategies in the identification and management of these risks. We also investigate the tools and techniques currently available to researchers during preclinical and clinical development of stem cell products, their utility and limitations, and how these tools may be strategically used in the development of these therapies. We conclude that ensuring safety through cutting-edge science and robust assays, coupled with regular and open discussions between regulators and academic/industrial investigators, is likely to prove the most fruitful route to ensuring the safest possible development of new products. STEM CELLS TRANSLATIONAL MEDICINE *2015;4:389–400*

INTRODUCTION

Stem cell therapies are moving rapidly into clinical application. Although it is important that these therapies are advanced into the clinic, their safety must be continually evaluated. Here we outline the known risks of stem cell therapeutics (supplemental online Fig. 1) and discuss how they can be assessed and managed through preclinical and clinical trials. This review is the output of an Innovative Medicines Initiative SafeSciMET workshop held at the University of Liverpool.

A key issue in the understanding of the safety concerns is the breadth of the human stem cell field, with several cell types falling under the umbrella term "stem cell":

- Human embryonic stem cells (hESCs) are pluripotent cells, first isolated from human embryos in 1998 by Thomson et al. [1].
- Human induced pluripotent stem cells (hiPSCs) were first reported in 2006. Somatic cells were reprogrammed using the transcription factors Oct4, Sox2, Klf4, and c-Myc (OSKM) to a pluripotent stem cell state [2, 3].
- Adult stem cells (ASCs) cover several cell types including mesenchymal and hematopoietic stem cells and tissue-specific progenitors that reside in the human body throughout an individual's life. In comparison with pluripotent stem cells, they generally have a more limited expansion and differentiation capacity [4, 5].

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Some adult stem cell-based therapies are clinically available, such as bone marrow or cord blood transplants containing hematopoietic stem cells [6, 7], skin grafts for burns [8], and mesenchymal stem cells for graft-versus-host disease (GVHD) in children (Canada and New Zealand) [9].

Additionally, more than 3,000 trials associated with stem cells are currently collated in the World Health Organization International Clinical Trials Registry Platform. The majority of these are adult stem cell-based therapies, likely attributable to the longer established use of these cells.

The registry also includes the first pluripotent-based therapies to be subjected to clinical trials; Table 1 highlights the narrow scope of these hESC/hiPSC-derived therapeutics, with 8 of the 9 treatments associated with macular dystrophy or degeneration, including the recently approved first human trial using hiPSCs [10]. Use of the eye as a first application of these cells is ideal: the graft size required is small, retinal pigment epithelial cells are easily differentiated to high purity, and the grafts can be visualized noninvasively, all contributing to a lower risk profile than hESC/hiPSC grafts in less accessible organs [11, 12]. Other iPSC-related trials listed on the registry are related to the generation of genotype- or disease-specific iPSC lines for use as disease/genotype models and stem cell banks, highlighting the broad appeal of hiPSCs.

Despite the basic technology being in place to produce a wider range of therapies, many aspects of the field, including safety, remain incompletely understood, contributing to the cautious translation from theoretical benefits to clinical application.

Stem Cell Risk Factors

Tumorigenic Potential

A major concern over the use of stem cell therapies is the perceived risk of tumorigenicity. This is exemplified by the investigation of a tumor that developed four years after fetal neural stem cell transplantation for ataxia telangiectasia [13]. Subsequent analysis found that the tumor was derived from the transplanted material. Similar cases have also been reported in the treatment of spinal injury with olfactory mucosal cell transplantation; following presentation with back pain 8 years after the treatment, the patient was found to have developed a mucosal-like mass at the transplant location [14]. This study is particularly pertinent given that the treatment used adult stem cells, which are often considered to be less tumorigenic than fetal or pluripotent stem cells, and the recent groundbreaking treatment of spinal injury with olfactory ensheathing cells [15]. In this study, the authors report no adverse effects after 19 months; however, tumors from stem cell grafts can arise many years after transplantation, highlighting the need for extensive follow-up programs to reduce patient risk.

The capacity for undifferentiated pluripotent stem cells to form teratomas in vivo is of particular concern [16]. Therefore, these cells will be differentiated before transplantation. However, the risk remains that not all cells will be fully differentiated. One study showed that despite functional liver engraftment, hESC-derived hepatocyte-like cells transplanted into immunocompromised mice developed splenic and liver tumors containing endodermal and mesodermal cell types [17]. Teratomas have also been shown to be able to form from as little as 0.2% SSEA-1-positive pluripotent cells, demonstrating that even at high levels of purity, teratoma formation potential remains [18].

It is therefore vital to prevent undifferentiated cells passing through to the differentiated cell population. Techniques to address this problem include small molecules targeting stearoyl-CoA desaturase-1, which selectively causes cell death in undifferentiated iPSC/ESCs [19]. However, current analytical techniques are not reliably sensitive enough to detect the removal of all pluripotent cells [20]. Therefore, it is important to take other factors, such as the disease and the number of cells transplanted, into account, because these factors will likely alter the chances of subsequent teratoma formation [21]. Recent work has alleviated some concerns; a nonhuman primate model for autologous transplants showed that iPSC-derived mesodermal stromal-like cells went on to form functional tissue, without teratoma formation [22].

Human studies are the only true way to ascertain the teratoma risk in man. The first human studies were conducted by Geron in 2009 [23], using hESC-derived oligodendrocyte progenitor cells for spinal injury treatment. The trials were halted for financial reasons, but in the few patients treated, no tumors have been reported [24]. Clinical trials investigating the use of hESC- and iPSC-derived retinal pigmented epithelial cells in macular degeneration are currently ongoing [11] and just starting [10], respectively, with no tumor formation reported as yet. If successful, these trials are likely to alleviate some of the concerns surrounding tumorigenesis from pluripotent stem cells.

Pluripotent cells can be cultured indefinitely in vitro, making scale-up relatively straightforward. However, during expansion the cells are susceptible to chromosomal aberrations and karyotype abnormalities [25–32], potentially because of the artificial conditions in which the cells are cultured, increasing the potential for post-transplant malignancy. Pioneering work has investigated these aberrations, commonly found at chromosomes 1, 12, 17, and 20, at higher resolution; however, it remains to be seen whether the "culprit" genes can be identified for screening [26–28, 30–36]. It is clear that smaller genomic changes also occur, often at a level not readily detected by standard G-banding [26]; the significance of these changes to safety is unclear. Much work has been focused on the removal of pluripotent stem cells from the transplanted material; however, techniques that allow for the removal for genotypically compromised cells would be of equal benefit to the therapeutic safety profile [37]. Karyotypical changes are not limited to pluripotent cells, with ASCs also thought to develop abnormalities during in vitro culture [34]; however, these findings have been debated, as demonstrated by the correspondence between Sensebé et al. [38] and Ben-David et al. [39].

iPSCs have additional safety concerns. The development of nonintegrative reprogramming techniques using direct transfection of proteins or mRNAs, Sendai viruses, or episomal plasmids has reduced concerns regarding incomplete promoter silencing and genomic disruptions of traditional techniques [40–43]. Some have also replaced the potentially oncogenic OSKM reprogramming factors with Sall4, Nanog, Esrrb, and Lin28 [44]; these factors are thought to be less efficient but derive higher quality iPSCs with reduced aberrations in histone variant 2A.X, which has been shown to be a key determinant of iPSC/ESC quality and developmental potential [45]. Others have used microRNAs and small molecules to reprogram somatic cells [46, 47]; however, at the time of writing, these reports are yet to be replicated.

Additional studies investigating the genomic integrity of iPSCs have shown that DNA damage sustained during reprogramming

Table 1. Pluripotent stem cells clinical trials (phases I–III) listed in the International Clinical Trial Registry Platform by the World Health Organization

ICTRP Trial	Disease	Cell type	Trail stage	Country	Financial support	Registration date (month/day/year)
NCT02122159	Myopic macular degeneration	hESC-derived retinal pigmented epithelial cells	I/II	USA	University of California, Los Angeles	4/1/2014
JPRN-UMIN000011929	Exudative age-related macular degeneration	hiPSC-derived retinal pigmented epithelial cells	I	Japan	RIKEN	10/2/2013
NCT02057900	Ischemic heart disease	hESC-derived CD15+ Isl-1+ progenitors	I	France	Assistance Publique-Hôpitaux de Paris	9/17/2013
NCT01691261	Acute wet age-related macular degeneration	hESC-derived retinal pigmented epithelial cells	I	USA/U.K.	Pfizer	9/19/2012
NCT01674829	Advanced dry age-related macular degeneration	hESC-derived retinal pigmented epithelial cells	I/II	South Korea	CHA Bio & Diostech	8/22/2012
NCT01625559	Stargardt's macular dystrophy	hESC-derived retinal pigmented epithelial cells	I	South Korea	CHA Bio & Diostech	6/18/2012
NCT01469832	Stargardt's macular dystrophy	hESC-derived retinal pigmented epithelial cells	I/II	U.K.	Advanced Cell Technology	11/08/2011
NCT01344993	Advanced dry age-related macular degeneration	hESC-derived retinal pigmented epithelial cells	I/II	USA	Advanced Cell Technology	4/28/2011
NCT01345006	Stargardt's macular dystrophy	hESC-derived retinal pigmented epithelial cells	I/II	USA	Advanced Cell Technology	4/28/2011

Abbreviations: hESC, human embryonic stem cell; ICTRP, International Clinical Trial Registry Platform.

may not be fully repaired in the resulting cells [48]. Furthermore, reprogramming cord blood cells reduced the number of DNA mutations when compared with dermal fibroblasts [49], suggesting that reprogramming from neonatal or more stem-like cells may be theoretically safer, albeit more challenging to obtain.

Immunogenic Potential

Maintaining functional immunologic tolerance of stem cells and their derivatives is crucial. Rejection is considered to be due to a mismatch in expression of human leukocyte antigens (HLA), minor histocompatibility complex (mHC) antigens, and ABO blood group antigens following allogeneic transplant (supplemental online Fig. 2). Generally, allogeneic matching for both HLA and mHC is not feasible because of extensive polymorphisms.

Undifferentiated ASC immunogenicity studies are particularly important, because, unlike pluripotent cells, they can be administered without differentiation. Mesenchymal stem cells (MSCs) have a unique capacity amongst ASCs to modulate the immune response through a HLA-independent [50] dampening of inflammatory cytokine release [51–53]. Additional low HLA-I and no extracellular HLA-II [51] alongside little or no expression of B- and T-cell costimulatory molecules [54, 55] on MSCs suggest a potential to both modulate and avoid immune surveillance.

Other ASCs, such as hematopoietic stem cells (HSCs), have also demonstrated some immune avoidance capabilities [56, 57], but allogeneic transplants are still susceptible to rejection [58]. Moreover, the vast experience with the use of allogeneic HSC transplants for the treatment of haematological malignancies and other conditions has shown the potential for GVHD as a result of allogeneic T-cell infiltration from the graft. This represents a major risk factor and cause of patient morbidity and mortality, with ∼15% of allogeneic HSC transplants resulting in fatalities [59]. This is a large and important topic that is well-reviewed by Blazar et al. [60]. Interestingly, MSCs have been used for the treatment of GVHD (Prochymal) [9, 61, 62]. This has led some to suggest that MSCs could be used as part of the stem cell transplant to reduce the potential for both GVHD and graft rejection [63].

Because of tumorigenic risk, clinical administration of pluripotent stem cells is likely to be in the form of a differentiated population; thus any immunogenic assessment should focus on the differentiated product [64]. It is generally accepted that there is little to no rejection in autologous cells, even following in vitro culture. Therefore, research has focused on developing stem cells, which are genetically identical to the recipient. Recently, somatic cell nuclear transfer was achieved in humans, allowing for the isolation of hESCs expressing the donor genotype [65, 66].

iPSC-based therapy remains the most promising technique for realizing pluripotent autologous therapy. Although initial reports suggested immunogenicity in syngeneic transplants [67], two subsequent studies found no evidence of acute or chronic immunogenicity toward differentiated iPSCs (both spontaneous and directed) [68, 69]. Further, de Almeida et al. [70] reported that, in contrast to rejected iPSCs, syngeneic iPSC-derived endothelial cells were accepted in mice, demonstrating a comparable tolerogenic response to syngeneic primary endothelial cells. Direct comparison of autologous and allogeneic transplanted iPSC-derived neurons in nonhuman primates also revealed minimal immune response in autologous transplants, whereas allogeneic transplants were immunogenic [71]. Therefore, current evidence points toward immunological tolerance of autologous terminally differentiated transplanted stem cells.

The time scale and costs associated with personalized therapies may mean that they are used as an alternative option when HLA matching cannot be achieved from stem cell banks containing carefully selected donor cell lines [72–74]. A second consideration is for disorders in which their etiology is genetically linked and whether patient-derived transplanted material containing the diseased genotype would have therapeutic efficacy; autologous cells in such cases may require gene therapy.

One method of dealing with the immune response to cell grafts is encapsulation [75, 76]. Encapsulation reduces interaction with immune cells and consequently reduces the risk of rejection

while maintaining efficacy through the movement of factors (e.g., cytokines) across a semipermeable membrane. Furthermore, encapsulation may also prevent tumors from reaching tissues outside the capsule. Such techniques are currently being developed for use in diseases such as diabetes and may represent an elegant solution to a complex problem [77–80]. Notwithstanding the clear potential, the development of such a system is not trivial, and despite sustained efforts and sequential developments, the translation to a clinically effective technology has yet to be achieved [81].

Another immunological consideration is the culture and manufacturing conditions. For example, fetal bovine serum and sialic acid derivative Neu5G from mouse feeder layers have both been shown to alter the immunogenicity of stem cells [82, 83]. Therefore, certified animal component-free products should be used wherever possible.

Biodistribution

Biodistribution encompasses the risks associated with the migration, distribution, engraftment, and long-term survival of the transplanted material. Different routes of administration result in differential dissemination patterns and risks. Systemic administration can lead to cells becoming entrapped in the lung or microvasculature, causing dangerous side effects, such as the pulmonary emboli reported following intravenous administration of adipose tissue-derived stem cells [84]. Administration in a feeding artery of the target tissue has been proposed to reduce these risks [85]; however, the risk of microvascular occlusions remain. Direct transplant to the targeted organ/area may reduce these risks [86, 87]; however, this is likely to be location-dependent and may require invasive surgery, for example, in the liver. Therefore, the chosen method must consider the target pathology, therapeutic objectives, and the patient risk-benefit profile [88, 89].

Once administered, up to 90% of transplanted cells are lost because of physical stress, inflammation, hypoxia, anoikis, or immunogenic rejection [20, 90]. To achieve therapeutic efficacy, large numbers of cells may therefore be required, increasing the risk of teratoma formation [21] or ectopic engraftment. Thus, the minimum number of cells required for effective treatment should be ascertained as part of product development.

A recent study of neural stem cells in a model of spinal cord injury reported ectopic cell growth 9–10 weeks post-transplant at various points along the spinal cord and brainstem [91]. The cells responsible for the ectopic growth were hypothesized to have travelled via the cerebral spinal fluid, colonized, and further proliferated, highlighting the need to understand the biodistributary properties of the treatment before clinical application.

The half-life of the transplanted material is another factor that can alter the level of risk. If the half-life is short, the risk associated with the transplanted material is reduced accordingly. However, if therapeutic efficacy is limited to the short-to-medium term, chronic diseases may require repeated administration and thus an understanding of the likely dosing regimen is another key consideration for risk assessment.

Regulation of Stem Cell Therapeutics

One of the major limitations of stem cell therapeutics is the heterogeneous character and limited experience of their development. Consequently, no specific European (European Medicines Agency [EMA]) or U.K. (Medicines and Healthcare Products Regulatory Agency, [MHRA]) regulatory guidance addresses technical aspects of the drug development program in detail, for example, the type, size, and duration of nonclinical studies [92].

Regulators have attempted to address these problems by drafting guidelines and reflection papers. The Guideline on Human Cell-Based Medicinal Products (EMEA/CHMP/410869/2006) was adopted in 2008, before the unifying regulation on advanced therapy and medicinal products came into force [93] and gives a generic overview of the requirements for the licensing of cell-based medicinal products; however, the information provided is not very detailed. A subsequent reflection paper on stem cell-based medicinal products (CAT/571134/09) was adopted in 2011, focusing more specifically on stem cell-based medicinal products and also discussing the experiences gained with cell-based products, including a summary of the challenges associated with biodistribution and immunogenicity studies. However, because no detailed requirements are defined, the applicant is still required to implement an appropriate development program that addresses the product-specific risks.

It is highly advisable to engage in discussions with the regulatory bodies early in the development of the product. Most regulatory agencies develop structures to facilitate the interaction with developers (e.g., the MHRA innovation office and the EMA innovation task force) and may provide scientific advice to assist product development.

For the development of advanced therapy medicinal products, a risk-based approach can be used as a matrix to decide that nonclinical data are needed. The (optional) risk-based approach encompasses intrinsic (cell-related) and extrinsic (manufacture-related) risks associated with the medicinal product and the subsequent development and implementation of the appropriate assays to assess these risks.

Further help with risk assessment is available in the Guideline on the Risk-Based Approach According to Annex I, Part IV of Directive 2001/83/EC Applied to Advanced Therapy Medicinal Products (EMA/CAT/CPWP/686637/2011). This document provides examples illustrating the risk-based approach. Likewise, (non-binding) guidance documents are also provided by the Food and Drug Administration (FDA) in the USA [94].

As a regulatory prerequisite, good manufacturing practice must also be followed, as well as the use of clinical grade stem cell products and procedures, free of microbiological and nonmicrobiological contaminants. Similar practices should be applied to preclinical research to allow predictable translation of therapies to the clinic.

The importance of regulation is highlighted by the report on the unregulated use of fetal brain-derived olfactory ensheathing cells for the treatment for spinal cord injuries. The authors found little to no benefit from the treatment, but complications including meningitis and death [95]. Although this is an extreme example, many unregulated stem cell treatments are now available across the world (well-reviewed by Zarzeczny et al. [96]). In 2011, Celltex began offering ASC-based therapies in Texas without FDA approval, igniting debate about the regulation of stem cell therapeutics [97]. Subsequently, the FDA won a recent court battle to regulate proliferated stem cells as biological drugs, and documents encapsulating these new regulatory powers are in preparation [98, 99].

Preclinical and Clinical Assessment

Tumorigenic and Immunogenic Preclinical and Clinical Trials/Assays

In terms of both tumor- and immunogenicity, risk cannot be reliably assessed when the model is not predictive, so it is important to match the targeted disease phenotype to the animal or in vitro assay. Traditional medicinal product development routes may be appropriate (i.e., going from simple to complex, in vitro to in vivo, and animal to human). However, some therapies may require multimodel studies to provide the fullest understanding of both efficacy and safety, whereas other therapies may not require an animal model because there may be little relevance. Future preclinical assessments may also use iPSC-derived cells as a source of a diseased phenotype as the most clinically relevant model of therapeutic safety and efficacy.

Assays for the Assessment of Tumorigenic Potential

The tumorigenic potential of cell-based therapies needs to be assessed throughout product development. *In vitro* techniques, such as karyotyping, can be used to assess genomic integrity. More in-depth investigation may be required to detect smaller changes; however, without known associated changes, attributing risk is difficult. Quantitative polymerase chain reaction (Q-PCR) and flow cytometry can be used to determine the purity of the differentiated population, and soft agar colony formation assays may also be used to assess the tumorigenic potential of the cell population [100]. However, all these indirect methods do not guarantee absence of tumors in the clinical setting.

Immune-deficient rodent models may be used to assess the direct tumorigenic potential of the transplanted material, with tumorigenic growth reported from as few as two undifferentiated ESCs [101]. Initial investigations may take place in an easily accessible and observable location with cell number determined by the planned assessment method. Once initial investigations are complete, tumorigenicity in the clinically relevant microenvironment should then be assessed with cell numbers equivalent to and higher than the predicted clinical dose. Deep tissue assessment by Q-PCR or histopathological analysis is usually required to confirm ectopic tumor formation [102, 103], but future investigations may use improvements in real-time cell tracking for greater information with regard to tumor location/development. Currently available imaging techniques suitable for clinical tumorigenic analysis include magnetic resonance imaging (MRI) for tumors $>$0.3 cm and fludeoxyglucose (18F) ([^{18}F]FDG)-positron emission tomography (PET) for tumors $>$1 cm, with bioluminescent and photoacoustic imaging currently limited to preclinical studies [104, 105]. The use of biomarkers in clinical trials may also provide useful information, with raised blood α-fetoprotein levels found in many teratomas [106]. Commonly used techniques for assessing tumorigenic potential in vitro and after clinical transplantation are presented in Table 2.

Immune-deficient models lack the immune response to tumor formation. Previous reports have demonstrated a reduced capacity for tumor formation in immune-competent models when compared with immune-deficient models [70, 101]. Consequently, a tumor that forms in an immune-deficient model may not always form in an immune-competent model or in clinical studies.

Preclinical nonxenogeneic studies using animal transplant models, as shown by Hong et al. [22] (e.g., transplanting equivalent mouse iPSC-derived cells into genetically identical/nonidentical mice) used in combination with in vitro assays before the development of human equivalents may therefore be the most relevant method of assessing tumorigenicity.

Assays for the Assessment of Immunogenic Potential

Developing relevant immunogenicity assays remains challenging. Immune-competent and immune-deficient in vivo models lack immunogenic clinical relevance for human cells in most situations; however, in some cases they can provide useful information:

- Immune-competent models may be used to investigate the use of stem cells in immune-privileged locations, such as the eye [12] or as a model of allogeneic transplants.
- Immune-deficient animals varying in the extent of immune depletion (i.e., loss of specific immune cell types) may be useful in investigating specific mechanisms of rejection [107].
- Humanized models, such as the trimera mouse, have human immune cells, improving relevance [108], especially for examining allogeneic grafts.

Recognizing that xenotransplation cannot capture the human alloimmune response [109], in vitro assays such as mixed lymphocyte reactions may be more informative of graft immunogenicity. Moreover, using the equivalent therapy in a species suitable for modelling immunogenicity, such as the nonhuman primate iPSC-derived transplant models reported by Morizane et al. [71], may provide the most informative results, if technically and financially viable.

Biodistribution in Preclinical and Clinical Trial/Assays

Biodistribution assays inform both safety and efficacy evaluations. Although histopathology and PCR remain the gold standard for assessing deep tissues, here we focus on cell labeling because of its ability to monitor cell distribution/migration in real time [110]. Such techniques are important for ascertaining the migratory/distribution patterns and are also informative in a tumorigenic (ectopic tumor formation) and immune (loss of cells through immune rejection) context.

Cellular imaging strategies are composed of the imaging technique and the labeling agent (supplemental online Fig. 3). The imaging technique is usually chosen in conjunction with the labeling agent, which can be classified in two main categories: direct and indirect labeling [111], summarized in Table 3.

Direct Labeling

Direct labeling requires the introduction of the labeling agents into the cells before transplantation. The relative intensity of the detected signal from the introduced molecules is then used as a surrogate for cell number.

Radionuclides used for cell imaging have different half-lives, which therefore determines the length of time cells can be monitored noninvasively [110]. Single photon emission computed tomography (SPECT) and/or PET are the most commonly used methods for detecting radionuclides (Table 3). Studies have shown as little as 6.2×10^3 to 2.5×10^4 cells can be detected using these methods [112]. However, short radionuclide half-lives mean that cell-tracking is limited to hours rather than weeks. Indium-111 oxine has a relatively long half-life ($\sim$2.8 days) [112] and has been shown to successfully track MSCs in preclinical models for up to 7

Table 2. Available assays to assess the tumorigenic risk of stem cell therapeutics, describing the main uses of each technique along with advantages and disadvantages

Assay	Intended use	Advantages	Disadvantages
Karyotyping (G-banding and/or spectral) [26, 28]	Assess genetic integrity	Unbiased genome coverage Can detect balanced translocations and inversions Cell-level resolution	Low genome resolution Low throughput
Comparative genomic hybridization arrays [27, 29, 30, 32]	Assess genetic integrity	High genome resolution Can probe specific zones	Does not detect changes in ploidy Unable to detect balanced translocations and inversions Population level resolution
Comparative large-scale expression analysis (e-karyotyping) [31, 34, 159]	Assess genetic integrity Assess cell differentiation	High genome resolution Can probe specific zones Expression profile and genetic integrity test at the same time	Indirect test for genetic integrity Does not detect changes in ploidy Unable to detect balanced translocations and inversions Population level resolution
Single-nucleotide polymorphism analysis [26, 29, 32]	Assess genetic integrity	High genome resolution Can probe specific zones	Does not detect changes in ploidy Unable to detect balanced translocations and inversions Population level resolution
Soft agar colony formation assay [100]	Assess colony formation in anchorage independent conditions	Well-established Relatively inexpensive	Not suitable for pluripotent cells that require "clump passage" Time consuming High limit of detection
Standard histology and cell microscopy [107, 160]	Assess cell differentiation	Cell-level resolution Can detect incomplete and immature phenotypes or transformation	Significant experience required Invasiveness for in vivo and clinical use Cannot discriminate between host and graft Low throughput
Standard molecular biology expression tools (northern and western blotting, ELISA, two-dimensional protein gels, PCR-related techniques) [28, 35, 161]	Assess cell behavior and differentiation	Can detect incomplete and immature phenotypes or transformation Can discriminate between host and graft (depending on technique and application)	Invasiveness for in vivo and clinical use Population level resolution
In situ hybridization and immunolabeling of endogenous transcripts/antigens (including bioluminescence and cell sorting techniques) [33, 162, 163]	Assess cell behavior and differentiation Cell preparation purification	Cell level resolution Combines histology and gene expression Can detect incomplete or immature phenotypes Can discriminate between host and graft (with adequate probe or antibody)	Invasiveness for in vivo and clinical use Low throughput
Mass spectrometry proteomics [164, 165]	Assess cell behavior and differentiation	High throughput Unbiased proteome coverage Can detect incomplete or immature phenotypes Can discriminate between host and graft (with labeling)	Significant experience required Sensitivity can be an issue for low abundance proteins Invasiveness for in vivo and clinical use
Standard toxicology studies [166]	Assess toxicity and tumor formation potential in animals and humans	Well-established Allows basic metabolic profiling of the host	Requires combined use of other techniques (i.e., histology, profiling, etc.)
Three-dimensional imaging techniques (MRI, CT, PET scans) [166, 167]	Assess tumor formation in animals and humans Assess status of graft/device Assess host status	Noninvasive Good spatial data Radioactive labeling (PET) can detect specific targets	Only morphological data (MRI and CT) Use of x-rays (CT) and/or radioactive reagents (PET) Requires expensive infrastructure
Photoacoustic imaging [135, 136]	Assess tumor formation in animals and humans	Noninvasive	Low skin penetration
Bioluminescence imaging [168]	Assess tumor formation in animals	Noninvasive	Low skin penetration

Abbreviations: CT, computed tomography; ELISA, enzyme-linked immunosorbent assay; MRI, magnetic resonance imaging; PCR, polymerase chain reaction; PET, positron emission tomography.

Table 3. Comparison of technologies for stem cell graft tracking in vivo

Strategy and imaging modality	Overview	Sensitivity, spatial resolution, duration of track	Advantages	Disadvantages
Direct cell labeling: MRI [110, 111, 126, 169, 170]	This technique is based on registration of change in electromagnetic properties of hydrogen atoms within a high-strength static magnetic field after a series of repetitive radiofrequency pulses and gradients.	10^{-3}–10^{-5} mol/liter 25–100 μm Cell lifetime (diluted over time)	High spatial and temporal resolution Combines functional and morphological visualization No exposure to ionizing radiation Clinically applicable Additional anatomical and pathological information	Signal dilution over time Low sensitivity No discrimination between live and dead cells May affect proliferation and cell morphology Long-term tracking is challenging Difficult quantification Requires large amount of contrast probe Accumulation of contrast probes can be toxic Needs expensive equipment
Direct cell labeling: Radionuclide imaging (PET and SPECT) [110, 111, 126, 169]	Ex vivo cellular uptake of radionuclides as a contrast agent (depending on the isotope used, the tracking period is different).	10^{-10}–10^{-12} mol/liter 1–2 mm Dependent on isotope half life	Picomolar sensitivity Good tissue penetration Translation to clinical applications	Leakage of radionuclides Limited time window Low spatial resolution Emission of ionizing radiation Signal dilution over time
Direct cell labeling: Optical fluorescence imaging [110, 171–173]	Cells are labeled ex vivo with QDs or fluorophores.	10^{-9}–10^{-12} mol/liter 2–3 mm 2–14 days (imaging), 8 weeks (QDs: histology)	High sensitivity High photostability (QDs)	Low resolution Limited tissue penetration No clinical application QDs potentially cytotoxic
Indirect cell labeling: Fluorescent imaging [167]	Cells are transduced with a gene that encodes for a fluorescent protein (GFP, RFP, etc.)	10^{-9}–10^{-12} mol/liter Up to 2 mm Cell lifetime	Longitudinal studies of stem cell viability No alteration of cell phenotype or differentiation capacity Controllable system	Genetic modification Not suitable in humans
Indirect cell labeling: Bioluminescence imaging [167, 174]	Cells are transduced with a bioluminescent reporter gene	10^{-15}–10^{-17} mol/liter 3–5 mm Cell lifetime	Reduced false positives High sensitivity Low costs Versatile	Genetic modification Not suitable for clinical use, unless with a combinatorial approach
Indirect cell labeling: Photoacoustic tomography [135, 136, 175, 176]	Cells are transduced with a gene that replies to photoacoustic waves with waves that are collected to produce a three-dimensional image. Gold nanoparticles can also be used.	10^{-11}–10^{-12} mol/liter (gold nanoparticles) Up to 7 cm Cell lifetime	Low scattering in tissues Multiscale high resolution imaging of biological structures 100% sensitivity Background-free detection Speckle-free	Genetic modification

This subject has been further reviewed by James and Gambhir [167].
Abbreviations: GFP, green fluorescent protein; RFP, red fluorescent protein; MRI, magnetic resonance imaging; PET, positron emission tomography; QD, quantum dot; SPECT, single photon emission computed tomography.

days [113]; however, signal leakage and alteration of cell phenotype limits translatability [114]. Clinically, hematopoietic stem cells labeled with [^{18}F]FDG for acute and chronic myocardial infarction treatment were successfully tracked by PET after 20 hours [115].

The use of iron oxide labeling for MRI makes it possible to trace the cells over longer periods of time [116]. The most common labeling agent in preclinical/clinical trials is superparamagnetic iron oxide particles (SPIO), which offers the highest sensitivity and has been used to track neural stem cells in a patient for up to 3 weeks [117]. Generally, MRI has lower sensitivity than SPECT/PET. The number of cells used for SPIO tracking in humans ranges from 3.71×10^5 to 17.4×10^6 cells [118], whereas de Vries et al. [119] were able to detect 1.5×10^5 dendritic cells in melanoma patients.

Alternatively, Perfluorcarbons (PFC) and Fluorine-19 (19 F) MRI can be used to track cells [120]. Cells are labeled with PFC emulsions before transplantation and subsequently detected as hotspots by 19 F MRI. The main advantage of this system is the low signal-to-noise ratio, caused by the low endogenous 19 F concentration, allowing for the quantification of cells at an estimated minimum sensitivity of 10^4 to 10^5 cells per voxel [120]. This system has been successfully exploited to monitor stem cell therapies [121–123] and is promising for clinical applications with some PFCs approved by the FDA [124]. This system has been applied clinically in dendritic cells, with a reported minimum sensitivity of 1×10^5 cells per voxel [125].

Indirect Labeling

Indirect labeling is the introduction of a reporter gene recognized by a corresponding probe or imaging system [20]. This system is highly controllable because only viable cells are able to transcribe the reporter gene [126].

In MRI-based gene reporter systems, the transduced gene is typically an intracellular metalloprotein (e.g., transferrin, ferritin, tyrosinase) which traps large quantities of iron in the cytoplasm for noninvasive detection [110, 126]. However, the trapped iron produces long-term background, which masks the viability of the cell [112]. Some have therefore suggested that the only transduced gene currently suitable for MRI cell tracking is lysine-rich protein [127]. In the SPECT and PET reporter gene imaging systems, a gene reporter (enzyme or receptor) requires an exogenously administered probe (tracer) to localize and quantify the stem cell product.

A number of groups successfully monitored ESCs [128] and MSCs [129, 130] in animal models, using gene reporter systems. These studies reported a reliable correlation in terms of localization, magnitude, and duration of the cells in vivo when compared with conventional methods (immunohistochemistry and PCR). The short half-life of the probes allows a defined continuous imaging period of no more than a few hours [128]. However, being noninvasive, monitoring of the stem cells at regular intervals was possible for up to 4 weeks [128–130]. Quantitative information can be extrapolated from the percentage of injected radioisotope/gram of tissue, allowing for the quantification of the area(s) covered by the cells, but not the exact cell number [129].

The use of indirect labeling is rare in a clinical setting because genetic manipulation is required [131]. However, the FDA has approved the PET reporter probe 9-[4-[^{18}F]fluoro-3-(hydroxymethyl) butyl]guanine ([^{18}F]FHBG; IND #61,880) [132] for the treatment of glioblastoma multiforme. Successful tracking of T cells was reported with no significant adverse effects [133]. Guidelines on how to administer and safely monitor [^{18}F]FHBG in humans have been made available [134].

Optical imaging techniques are limited by exponential signal loss as depth increases, caused by scattering phenomena that occur when photons pass through tissue [110, 126]. Photoacoustic tomography overcomes this problem. A short laser pulse irradiates the target tissue, causing a partial absorption of the pulse energy and conversion into heat. This increases local pressure through thermoelastic waves and is subsequently detected by ultrasonic transducers placed outside the tissue. The image is generated by collecting all thermoelastic waves from the arrival time [135, 136]. Such technology has been used to track human MSCs labeled with gold nanocages in a rodent model for 7 days [104].

Other Risks Associated With the Translation to the Clinic

Despite highly controlled conditions in both cell preparations and clinical settings, infections remain a risk for patients who have received allogeneic stem cell transplants that require immunesuppression therapy [137]. Moreover, long-term immunosuppression has well-documented side effects, including end-organ toxicity and increased risk of cancers [138].

Viral status must also be assessed in donors of allogeneic grafts. Donors of HSCs are routinely screened for hepatitis viruses, human immunodeficiency virus, cytomegalovirus, and (bacterial) syphilis [139, 140]. Further screening for herpes simplex virus, Epstein-Barr virus, and adenoviruses may also be required in addition to screening for cell type- and location-specific viruses [140]. Genotype screening for donor cells has also been suggested [141], with some reports of specific genetic polymorphisms associated with differential GVHD severity and outcome in allogeneic HSC transplants [142, 143].

Scaffolds aiding engraftment or delivery of cells should also be considered for immunological potential. Such devices have been used to improve the survival of MSCs in brain injury models [144, 145], and some groups are attempting to use decellularized organs [146] as three-dimensional scaffolds for stem cell-derived repopulation [147–149]. Biological scaffolds offer greater similarity to the host extracellular matrix than those of synthetic origin, improving engraftment; however, they are usually xenogeneic/allogeneic [150] and thus have immunogenic potential. Various techniques have been used to remove antigenic epitopes, DNA, and damage-associated molecular pattern signals [151–154]; however, immunogenic potential remains. A comparative study of five commercially available biological scaffolds demonstrated significantly elevated immune responses, including chronic inflammation and fibrosis, versus an autologous control [155].

Scaffolds derived from synthetic origin are generally considered to be less immunogenic. Several synthetic biodegradable polymers have been approved by the FDA for medical applications [156–158] and consequently may be used without further safety assessment. However, novel materials/uses are required to undergo safety testing in compliance with the ISO 10993 International Standard (ISO 10993: Biological evaluation of medical devices).

CONCLUSION

Stem cell therapies have immense potential to alleviate, or even cure, a range of acute, chronic, and debilitating diseases.

However, we must ensure that these therapies are safe as well as effective, and a lot of work still remains to be done to understand and reduce any risk associated with their use.

Huge improvements in our in vitro techniques are needed, such as ensuring gene aberration-free expansion and improved differentiation purity, alongside the better identification of risk factors that can be routinely screened before transplantation. Furthermore, the development of models that can better predict immunological responses and cell tracking techniques with increased duration and depth capabilities would represent great improvements to the current status quo.

However, the top priority is that this work must remain focused on the clinical outcome. The most important consideration is the risk-benefit assessment for the patient. Although a stem cell therapy, like many drugs, may not be perfectly safe, the benefit to the patient may far outweigh the potential risks. Therefore, each treatment should be determined on a case-by-case basis with regulatory input, ensuring that the risk of the therapy is appropriate for the given condition and patient.

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Author Contributions

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Disclosure of Potential Conflicts of Interest

T.G.H. has compensated employment from the University of Basel and compensated research funding as an Integrative Kidney Physiology and Pathophysiology Marie Curie Postdoctoral Fellowship and from the Swiss National Centre for Competance in Research. R.B. has compensated research funding from the University of Liverpool. E.I.G. has compensated employment. B.P.M. is a compensated employee of Almirall S.A. P.W.A. has royalty payments by the Wistar Institute on receipts from income arising from nonexclusive licencing of hybridomas owned by Wistar and originally produced by P.W.A.: the hybridomas include those producing antibodies to hESC marker antigens, TRA-1-60, TRA-1-81, TRA-2-54, and TRA-2-49 and uncompensated research funding as partial payment of Ph.D. student stipend and research costs by Astra Zeneca. D.C.H. has uncompensated employment with CSO Fibromed Products Ltd. and uncompensated stock options with CSO Fibromed Products Ltd. M.E.S. is employed by the Cell Therapy Catapult. G.S.'s spouse is an employee of Stem Cell Sciences Ltd. P.B. is an employee of Cellectis AB and owns shares in Cellectis AB. J.P. is employed by and has shares in GlaxoSmithKline and has compensated honoraria for train travel and hotel accommodation from Society for Endocrinology as a speaker at an annual conference. J.M. is employed by and has shares in AstraZeneca Pharmaceuticals. D.P.W. is an employer. The other authors indicated no potential conflicts of interest.

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Concise Review: Workshop Review: Understanding and Assessing the Risks of Stem Cell-Based Therapies

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