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.

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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 these 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 pre-clinical 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 are likely to prove the most fruitful route to ensuring the safest possible development of new products.

Abbreviations: (human/murine) induced pluripotent stem cell, (h/m)iPSC; (human/murine) embryonic stem cell, (h/m)ESC; mesenchymal stem cell, MSC; hematopoietic stem cells, HSCs; adult stem cell, ASC; graft versus host disease, GVHD; major histocompatibility complex; MHC; minor histocompatibility complex, mHC; Oct4, Sox2, Klf4, c-Myc, OSKM; human leukocyte antigen, HLA; Magnetic Resonance imaging, MRI; computed tomography, CT; positron emission tomography, PET; single photon emission computed tomography, SPECT; superparamagnetic iron oxide particles, SPIO; 9-[4-[18F]Fluoro-3-(hydroxymethyl)butyl]guanine, [18F]FHBG; perfluorocarbon, PFC; fluorine-19, 19 F; single nucleotide polymorphism, SNP; Food and drug administration, FDA; Medicines and healthcare products regulatory agency, MHRA. QD, quantum dots; G/RFP, green or red fluorescent protein
I. Introduction

The field of stem cell therapeutics is moving towards widespread clinical application. Whilst it is vital that this development continues, the safety of these therapies must also be considered. Here we outline the known risks of stem-cell therapeutics (Figure S1) and discuss how they can be assessed and managed through preclinical and clinical trials. This review is the output of an IMI SafeSciMET workshop held at the University of Liverpool.

II. Stem cell risk factors

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 of ‘stem cell’:

- **Human embryonic stem cells (h)ESCs** are pluripotent cells, first isolated from human embryos in 1998 by James Thompson.
- **Induced pluripotent stem cells (hiPSCs)** were first reported in 2006 by Shinya Yamanaka, demonstrating the reprogramming of somatic cells from mice and later humans, using 4 transcription factors: Oct4, Sox2, Klf4 and c-Myc (OSKM), to a pluripotent stem cell-like state.
- **Adult stem cell (ASCs)** covers several cell types including mesenchymal and hematopoietic stem cells and tissue-specific progenitors which reside in the human body throughout an individual’s life and in comparison to pluripotent stem cells, generally have a more limited expansion and differentiation capacity.

Some adult stem cell-based therapies are clinically available, such as bone marrow transplants containing hematopoietic stem cells, skin grafts for burns patients, cord blood cells for blood disease therapies and mesenchymal stem cells for graft vs host disease (GVHD) in children (Canada and New Zealand).

Additionally, over 3000 trials associated with stem cells are currently collated in the international clinical trial registry platform (www.who.int/trialsearch). 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-derived therapeutics, with 8 of the 9 treatments associated with macular dystrophy or degeneration. Moreover, the first human trial using hiPSCs has recently been approved for macular degeneration therapy. This is due to the high retinal epithelial cell differentiation purity and the ease of access to the eye for treatment reducing the risk profile of the treatment, making it an ideal starting point for hESC/hiPSC therapies.

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.

II.I 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 brain tumor which had developed in a child four years after being
treated with fetal neural stem cells for ataxia telangiectasia\textsuperscript{13}. Subsequent analysis found that the tumor was derived from the transplanted material, thus demonstrating the risks which are associated with stem cell-based therapies.

The capacity for undifferentiated pluripotent stem cells to form teratomas \textit{in vivo} is of particular concern\textsuperscript{14}. Therefore, it is unlikely that any therapy would directly transplant undifferentiated pluripotent stem cells, with \textit{in vitro} differentiation the most likely route 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\textsuperscript{15}. 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\textsuperscript{16}.

It is therefore vital to prevent undifferentiated cells passing through to the differentiated cell population. Such techniques include small molecules targeting stearoyl-CoA desaturase-1, which selectively causes cell death in undifferentiated iPSC/ESCs\textsuperscript{17}. However, the removal of all pluripotent cells cannot be confirmed with current analytical techniques as these are not reliably sensitive enough\textsuperscript{18}. Therefore, it is important to take other factors, such as the disease and the number of cells transplanted into account, as this will likely alter the chances of subsequent teratoma formation. Recent work has alleviated some concerns, a non-human primate model for autologous transplants showed that iPSC-derived mesodermal stromal-like cells went on to form functional tissue, without teratoma formation\textsuperscript{19}.

Human studies are the only true way to ascertain the teratoma risk in man. The first human studies were conducted by Geron in 2009, using hESC-derived oligodendrocyte progenitor cells for spinal injury treatment\textsuperscript{20}. Despite the trials having to be halted for financial reasons, no subsequent reports of tumor formation have emerged in those who were treated\textsuperscript{21}. Clinical trials investigating the use of hESC- and iPSC-derived retinal pigmented epithelial cells in macular degeneration are currently ongoing\textsuperscript{11} and just starting\textsuperscript{10}, respectively, with no tumorigenic safety concerns reported as yet. If successful, these trials are likely to alleviate some of the tumorigenic concerns surrounding pluripotent stem cells.

Pluripotent cells can be cultured indefinitely \textit{in vitro}, making scale-up production relatively straightforward. However, during expansion the cells are susceptible to chromosomal aberrations and karyotype abnormalities\textsuperscript{22–29}, potentially due to 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 if the ‘culprit’ genes can be identified for screening\textsuperscript{23–25,27–33}. It is clear that smaller genomic changes also occur, often at a level not readily detected by standard G-banding\textsuperscript{23}; 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 which allow for the removal for genotypically compromised cells would be of equal benefit to the therapeutic safety profile\textsuperscript{34}. Karyotypic changes are not limited to pluripotent cells, with ASCs also thought to develop abnormalities during \textit{in vitro} culture\textsuperscript{31}; however, these findings have been debated, as demonstrated by the correspondence between Sensebe \textit{et al.}\textsuperscript{35} and Ben-David \textit{et al.}\textsuperscript{36}. 
iPSCs have additional safety concerns. The development of non-integrative reprogramming techniques, utilizing direct transfection of proteins or mRNAs, Sendai viruses or episomal plasmids, has reduced concerns regarding incomplete promoter silencing and genomic disruptions of traditional techniques. Some have also replaced the oncogenic-associated OSKM factors with Sall4, Nanog, Esrrb, and Lin28; 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. Others have utilized microRNAs and small molecules to reprogram somatic cells; however, at the time of writing, these reports are yet to be repeated.

Additional studies investigating the genomic integrity of iPSCs have shown that DNA damage sustained during reprogramming may not be fully repaired in the resulting cells. Furthermore, reprogramming cord blood cells reduced the number of DNA mutations when compared to patient-derived dermal fibroblasts, suggesting that reprogramming from neonatal material may be theoretically safer, albeit more challenging to obtain.

II. II Immunogenic potential

Maintaining immunological tolerance of stem cell transplants 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 (Figure S2). Generally, allogeneic matching for both HLA and mHC is not feasible due to extensive polymorphisms.

Undifferentiated ASC immunogenicity studies are particularly important, as, unlike pluripotent cells, they can be administered without differentiation. MSCs have a unique capacity amongst ASCs to modulate the immune response through a HLA-independent dampening of inflammatory cytokine release. Additional low HLA-I and no extracellular HLA-II alongside little or no expression of B- and T-cell co-stimulatory molecules on MSCs, suggest a potential to both modulate and avoid immune surveillance.

Hematopoietic stem cells (HSCs) have also demonstrated some immune avoidance capabilities. However, HSCs are known to be susceptible to GVHD and can be rejected, representing a major cause of patient morbidity and mortality. Interestingly, MSCs have been used for the treatment of GVHD (Prochymal). This has led some to suggest that MSCs could be used as part of the stem cell transplant to reduce the potential for graft rejection and has been shown to reduce T cell activation in animal models.

Due to 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 resulting cell-type. It is generally accepted that there is little to no immune 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. However, with mitochondrial DNA from the oocyte unaltered, complete mHC compatibility is unlikely, meaning that immunosuppressant therapy may be required.

iPSC-based therapy remains the most promising technique to realizing pluripotent autologous therapy. Whilst initial reports suggested immunogenicity was still seen in syngeneic transplants, two
subsequent studies found no evidence of acute or chronic immunogenicity towards differentiated iPSCs (both spontaneous and directed) when the cells were syngeneically administered to mice\textsuperscript{64, 65}. Further, de Almeida \textit{et al.}, reported that, in contrast to rejected iPSCs, autologous iPSC-derived endothelial cells were accepted in mice, demonstrating a comparable tolerogenic response to primary endothelial cells\textsuperscript{66}. Direct comparison of autologous and allogeneic transplanted iPSC-derived neurons in non-human primates also revealed minimal immune response in autologous transplants; whereas allogeneic transplants were immunogenic\textsuperscript{67}. Therefore, current evidence points towards immunological tolerance of autologous terminally differentiated transplanted stem cells.

The timescale 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\textsuperscript{68-70}. 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; such cases may require gene correction technology, if HLA-type cannot be matched, as shown to be possible in monogenic conditions such as alpha-1-antitrypsin deficiency\textsuperscript{71}.

One emerging method of overcoming these issues is through encapsulation of the transplanted cells\textsuperscript{72, 73}. This may reduce the risk of tumor formation and immune rejection, whilst maintaining efficacy through the movement of factors (e.g. cytokines) across a semi-permeable membrane. Such techniques are currently being developed for use in diseases such as diabetes and may represent an elegant solution to a complex problem\textsuperscript{74-77}. 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\textsuperscript{78}.

\textbf{II.III Biodistribution}

Biodistribution encompasses the migration, distribution, engraftment and long term survival of the transplanted material.

Different routes of administration result in differential dissemination patterns; therefore, the appropriate method must be chosen, considering the target pathology and the therapeutic objectives\textsuperscript{79, 80}. Systemic administration can lead to cells becoming entrapped in the lung or microvasculature, causing dangerous side-effects, such as the pulmonary embolisms reported following intravenous administration of adipose-tissue derived stem cells\textsuperscript{81}. Therefore, the use of vasodilators\textsuperscript{82} or the administration in an artery close to the target tissue\textsuperscript{83}, have been proposed to reduce these risks.

However, arterial administration may still cause microvascular occlusions and thus, where possible, the ideal delivery method would be directly to the targeted organ/area\textsuperscript{84, 85}. Such techniques allow for maximal cell delivery and therapeutic outcome; however, in organs such as the liver or pancreas, the invasive nature of the direct cell transplantation may require thorough risk-benefit assessments.

Successful delivery may result in just 10\% viability after transplantation due to physical stress, inflammation, hypoxia or immunogenic rejection\textsuperscript{19}, meaning that very high numbers of cells may be required to achieve therapeutic benefit. As cell number increases, so does the potential for engraftment outside of the targeted tissues. It is therefore important to identify the cell location in
order to fully assess the consequences of ectopic engraftment. A recent study of neural stem cells in a rat model of spinal cord injury showed ectopic engraftment 9-10 weeks post-transplant at various points along the spinal cord and brainstem\textsuperscript{86}. Due to their size these were hypothesised to have travelled in small numbers via the cerebral spinal fluid, colonized and further proliferated, highlighting the concerns regarding ectopic engraftment, even in a direct transplant model.

The half-life of the transplanted material is another factor which can alter the level of risk. If short, the risk associated with the transplanted material is reduced accordingly. However, if the transplant does not have a capacity for long term survival and thus suffers from a loss of efficacy, chronic diseases may require repeated administration and thus an understanding of the likely dosing regime is another key consideration for risk assessment.

**III. Pre-clinical and clinical assessment**

One of the major limitations of stem cell therapeutics is the heterogeneous character and limited experience of their development. Consequently, there is currently no specific European (European Medicines Agency, EMA) or UK (Medicines and Healthcare Products Regulatory Agency, MHRA) regulatory guidance\textsuperscript{87} that addresses technical aspects of the drug development program in detail, e.g. type, size and duration of non-clinical studies.

Regulators have attempted to address these problems by drafting guidelines and reflection papers, whenever the necessity becomes apparent. The “Guideline on human cell-based medicinal products (EMEA/CHMP/410869/2006)” was adopted in 2008, before the unifying regulation on advanced therapy a medicinal product came into force\textsuperscript{88}, and gives a generic overview on 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 discusses the experiences gained with cell-based products, including a summary of the challenges associated with biodistribution and immunogenicity studies. However, since 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 that any institution aiming to initiate the development of a new product engages in early, open discussions with the regulatory bodies. 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 based on the concepts and already existing data to assist the product development process.

For the development of advanced therapy medicinal products, a risk-based approach can be used as a matrix to decide which non-clinical data are needed based on the risk of the medicinal product. This is an optional pathway determined by the product developer, which encompasses the identification of the potential 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.
This is further outlined 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). The annex of the guideline provides some non-exhaustive examples to better illustrate this concept. Likewise, (non-binding) guidance documents are also provided by the Food and Drug Administration (FDA) in the USA.  

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 and complications, including meningitis and reports of patient mortality. Whilst this is an extreme example, many unregulated stem cell treatments are now available across the world (well reviewed by Zarzeczny et al., 2019). In 2011, Celltex® began offering ASC-based therapies in Texas, USA without FDA approval, igniting debate about the regulation of stem cell therapeutics. Subsequently, the FDA has won a recent court battle to regulate proliferated stem cells as biological drugs and documents encapsulating these new regulatory powers are in preparation.

### III.1 Tumorigenic and immunogenic pre-clinical and clinical trials/assays

In terms of both tumor- and immunogenicity, risk is increased 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 multi-model studies to provide the fullest understanding of both efficacy and safety, whilst other therapies may not require an animal model as there may be little relevance. Future pre-clinical assessments may also use iPSC-derived cells as a source of a diseased phenotype as the most clinically relevant assay of therapeutic safety and efficacy.

#### Tumorigenicity assays

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 with regard to duplications, translocations and other chromosomal aberrations. More in-depth investigations may be required detect smaller changes; however, without known associated changes, attributing risk is difficult. Immune-deficient rodent models may be used to assess the tumorigenic potential of the transplanted material. Deep tissue assessment by q-PCR or histopathological analysis is usually required to confirm ectopic tumor formation, but future investigations may utilize improvements in real-time cell tracking for greater information with regard to tumor location/development, particularly in clinical trials. These techniques are evaluated in table 2.

Additionally, a recent study showed that whilst teratomas were formed in immune-deficient models, immune-competent models rejected autologous iPSCs; therefore formation of a human iPSC-derived teratoma in an immune-deficient pre-clinical model may not always translate to the clinical situation. The xenogeneic nature of such transplants may consequently require pre-clinical studies using syngeneic/allogeneic species-specific transplants before the development of human equivalents.

#### Immunogenicity assays
Developing relevant immunogenicity assays remains challenging. Early in product development, using the equivalent therapy in a different species for autologous or allogeneic investigations, as shown by Morizane et al\textsuperscript{67}, may provide the most informative results, if technically and financially viable.

Immune-competent and immune-deficient \textit{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\textsuperscript{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\textsuperscript{97}.
- Humanized models, such as the trimera mouse, have human immune cells, improving relevance\textsuperscript{98}. However, such models would again be limited to allogeneic investigations.

It is important to recognize that species differences and xenogeneic transplantation are likely to cause species-dependent translational issues in all discussed \textit{in vivo} models\textsuperscript{99}, potentially making \textit{in vitro} assays, such as mixed lymphocyte reactions, more informative of the final human-based product.

\textbf{III.II Biodistribution in pre-clinical and clinical trial/assays}

Biodistribution informs both the efficacy and safety of the treatment. Whilst histopathology and PCR remain the gold standard for assessing deep tissues, here we focus on cell labelling due to its ability to monitor cell distribution/migration in real-time\textsuperscript{100}. Such techniques are important for ascertaining the migratory/distribution patterns and are also informative in a tumorigenic (ectopic tumor formation) and immunogenic (loss of cells through immune rejection) context.

Cellular imaging strategies are composed of the imaging technique and the labelling agent (figure S3). The imaging technique is usually chosen in conjunction with the labelling agent, which can be classified in two main categories: direct and indirect labelling\textsuperscript{101}, summarized in table 3.

\textit{Direct Labelling}

Direct labelling requires the introduction of the labelling agents into the cells before transplantation. The number of molecules introduced into the cell is then used as a surrogate for cell number.

Radionuclides used for cell imaging have different physical half-lives, determining the length of time cells can be monitored non-invasively\textsuperscript{100}; these are mainly detected using single photon emission computed tomography (SPECT) and/or positron emission tomography (PET; table 3). Studies have shown as little as $6.2 \times 10^3$-$2.5 \times 10^4$ cells can be detected using these methods\textsuperscript{102}. 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)\textsuperscript{102} and has been shown to successfully track MSCs in preclinical models for up to 7 days\textsuperscript{103}; however, signal leakage and alteration of cell phenotype limits translatability\textsuperscript{104}. Clinically, hematopoietic stem cells labelled with $^{18}$F-FDG for acute and chronic myocardial infarction treatment were successfully tracked by PET after 20 hours\textsuperscript{105}.

The use of iron oxide-labelling for MRI is non-ionizing and makes it possible to trace the cells over longer periods of time\textsuperscript{106}. The most common labelling agent in pre-clinical/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\textsuperscript{107}. Generally, MRI has lower sensitivity than SPECT/PET. The number of cells used for SPIO tracking in man ranges from $3.71 \times 10^5$ to $17.4 \times 10^6$ cells\textsuperscript{108} whilst de Vries \textit{et al} were able to detect $1.5 \times 10^5$ dendritic cells \textit{in vivo}\textsuperscript{109}.

Alternatively, Perfluorcarbon (PFC) probes and contrast agent Fluorine-19 (19 F) can be used to label cells\textsuperscript{110}. The low signal-to-noise ratio and the absence of background make the quantification of pool of cells feasible. The amount of 19 F typically varies between $10^{11}$ and $10^{13}$ per cell, potentially dependent on the cytoplasmic volume. Empirically, it has been estimated that the minimum sensitivity of cell detection would be $10^4$-$10^5$ cells per voxel (value in a tri-dimensional grid)\textsuperscript{110}. This system has been successfully exploited to monitor stem cells therapies\textsuperscript{111-113} and are promising for clinical applications\textsuperscript{110} with some PFC and Fluorine-19 approved by the FDA\textsuperscript{114}.

\textit{Indirect Labelling}

Indirect labelling is the introduction of a reporter gene encoding for a fluorescent protein or a product recognizable by a reporter probe\textsuperscript{18}. This system is highly controllable because only viable cells are able to transcribe the reporter gene\textsuperscript{115}.

In MRI-based gene reporter systems, the transduced gene is typically an intracellular metalloprotein (e.g. transferrin, ferritin, tyrosinase), that traps large quantities of iron in the cytoplasm for non-invasive detection\textsuperscript{100,115}. However, the trapped iron produces long-term background which masks the viability of the cell\textsuperscript{102}. Some have therefore suggested that the only transduced gene currently suitable for MRI cell tracking is Lysine-rich protein\textsuperscript{116}.

In the SPECT and PET reporter gene imaging systems, a gene reporter (enzyme or receptor) requires an exogenously administered probe (tracer) to allow the localization and quantification of the stem cell product.

The most commonly used PET/SPECT reporter gene systems are:

- Intracellular enzymes (e.g. herpes simplex virus 1 thymidine kinase) (PET/SPECT).
- Mutant form of a dopamine receptor, a cell membrane protein that binds the radionuclide probe (3-(2’-[18F]-fluoroethyl)-spiperone)(PET).
- Sodium-iodide symporter, a thyroid transmembrane protein, which transports iodine into the cell (PET and SPECT).

A number of groups successfully monitored ESCs\textsuperscript{117} and MSCs\textsuperscript{118,119} 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 to 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\textsuperscript{117}. However, being non-invasive, monitoring of the stem cells at regular intervals was possible for up to 4 weeks\textsuperscript{117-119}. 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\textsuperscript{118}.

The use of indirect labelling is rare in a clinical setting as genomic alterations are required\textsuperscript{120}. However, the FDA has approved the PET reporter probe 9-[4-[18F] Fluoro-3-(hydroxymethyl) butyl]guanine ([18F]FHBG; IND #61,880)\textsuperscript{121} for the treatment of grade IV of glioblastoma multiforme. The group
successfully tracked the T-cells and also reported [18F]FHBG accumulation in the cytolytic T-cells\textsuperscript{121}, with no significant adverse effects\textsuperscript{122}. Guidelines on how to administer and safely monitor 18F-FHBG in humans have been made available\textsuperscript{123}.

Optical imaging techniques are limited by exponential signal loss as depth increases, caused by scattering phenomena that occur when photons pass through the tissue\textsuperscript{100, 115}. 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 thermo-elastic waves and is subsequently detected by ultrasonic transducers placed outside the tissue. The image is generated by collecting all thermo-elastic waves from the arrival time\textsuperscript{124, 125}. Such technology has been used to track human MSCs labeled with gold nanocages in a rodent model successfully for 7 days\textsuperscript{126}.

### III. Other risks associated with the translation to the clinic

Stem cells are not static products; consequently, cell culture and manufacturing conditions may introduce immunogenic alterations. For example, fetal bovine serum and sialic acid derivative Neu5G, found in stem cell culture conditions, have both been shown to alter the immunogenicity of stem cells\textsuperscript{127, 128}. Therefore, certified animal component-free products should be used wherever possible.

Good Manufacturing Practice must also be followed, as well as microbiological control of clinical grade stem cell products and aspects of viral safety and other contaminants. Similar practices should be applied to pre-clinical research in order to allow predictable translation of therapies to the clinic. Despite highly-controlled conditions in both cell preparations and clinical settings, infections (commonly respiratory-related) remain a risk for patients who have received allogeneic stem cell transplants which require immune-suppression therapy\textsuperscript{129}. Further, the donor viral status must also be assessed, with screening for hepatitis and HIV commonplace in HSC transplants\textsuperscript{130}.

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\textsuperscript{131, 132} and some groups are attempting to use decellularized organs\textsuperscript{133} as 3D scaffolds for stem cell-derived repopulation\textsuperscript{134-136}. Biological scaffolds offer greater similarity to the host extracellular matrix, improving engraftment; however, they are usually xenogeneic/allogeneic in origin\textsuperscript{137} and thus have immunogenic potential. Various techniques have been used in an attempt to remove/mask antigenic epitopes, DNA and damage-associated molecular pattern signals\textsuperscript{138-141}. However, a comparative rodent study demonstrated that across 5 commercially available scaffolds, differential immunogenic responses were found, including chronic inflammation and fibrous tissue, all of which differed from an autologous control\textsuperscript{142}.

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\textsuperscript{143-145}, and consequently may be used in the same site 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).

### IV. Conclusions
Stem cell therapies have the potential to offer alleviation from a range of chronic and debilitating diseases. Despite continued advances, much work remains in understanding and reducing the risks associated with stem cell therapeutics.

Improvements in in vitro techniques are required, such as gene aberration-free expansion and improved differentiation purity alongside the identification of risk factors which can be routinely screened before transplantation. Further, models which can better predict immunological responses and cell imaging techniques with increased duration and depth capabilities would also be beneficial.

However, this work must remain focused on the clinical outcome. The most important consideration is the risk-benefit assessment for the patient as whilst the cells, like many drugs, may not be perfectly safe, the patient benefit 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.

Acknowledgements

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**Figure legends**

**Figure S1: Therapeutic risks of stem cells.** Diagram demonstrating the main inherent safety concerns associated with stem cell therapeutics. These can be divided into 3 main categories: biodistribution: cell migration, distribution, engraftment and long-term survival; immunogenicity: graft-vs-host disease and other inflammatory/fibrotic conditions; tumorigenicity: genomic aberrations or insertions, cell purity (i.e. transplanted population containing iPSCs/ESCs with inherent teratoma potential) and cell of origin (i.e. the reduced risk of tumorigenicity with ASCs compared to iPSCs/ESCs, ESCs compared to iPSCs, and neonatal compared with adult cell-derived iPSCs).

**Figure S2: Schematic demonstrating the described potential mechanisms of immune recognition and rejection of stem cell grafts.** (1) **MHC-I incompatibility.** CD8+ve cytotoxic T-cells recognize the MHC-I as non-self (e.g. allogeneic transplants) and with additional detection of co-stimulatory molecules, elicit an immunogenic response leading to rejection. (2) **MHC-II incompatibility.** MHC-II-expressing antigen-presenting cells, present to CD4+ve T helper cells resulting in cytokine-induced inflammation and/or activation of B or T cell responses. (3) **Minor histocompatibility complex (mHC) incompatibility.** A selection of proteins expressed in the cell, including mitochondria derived proteins, may bind to and be presented by MHC-I. These can be recognized as mHC antigens, and lead to immune-rejection. (4) **ABO blood group antigen incompatibility.** ABO blood group antigens can be detected by antibodies and activate the complement system. (5) **Natural killer (NK) cell rejection.** When bound to the MHC-I molecule, NK cell binding to NK cell lysis receptors and subsequent cell-
killing is inhibited. In the absence of MHC-I, NK cells are free to bind to NK cell lysis receptors, and consequently elicit cell-killing. Undifferentiated stem cells are generally considered not to express MHC-II, NK cell lysis receptors NKp30, NKp46, CD16 and NKp44 or co-stimulatory molecules CD40, B7.1 and B7.2. Furthermore, stem cells are only considered to express low levels of MHC-I, although expression can be induced by IFN-γ. Expression of all of these molecules on the fully differentiated derivative however, is an important consideration when considering immunogenic tolerance.

**Figure S3: Stem cell imaging techniques.** Representative diagram of the two main cell imaging strategies: direct labelling and indirect labelling. Direct methods require the labelling of the cell with a non-integrative product, such as quantum dots, which reside in the cytoplasm and can be detected via MRI, PET, SPECT or fluorescent imaging depending on the technique. Indirect labelling requires a genetic modification of the cell, through the insertion of a gene reporter, which is then detected by the appropriate imaging technique. Details of each technique are listed in table 3.

<table>
<thead>
<tr>
<th>ICTRP Trial ID</th>
<th>Disease</th>
<th>Cell type</th>
<th>Trail stage</th>
<th>Country</th>
<th>Financial support</th>
<th>Registration date</th>
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<td>NCT02122159</td>
<td>Myopic Macular Degeneration</td>
<td>hESC-derived Retinal Pigmented Epithelial cells</td>
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<td>NCT02057900</td>
<td>Ischemic Heart Disease</td>
<td>Human Embryonic Stem Cell-derived CD15+ Isl-1+ Progenitors</td>
<td>I</td>
<td>France</td>
<td>Assistance Publique - Hôpitaux de Paris</td>
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<tr>
<td>NCT Number</td>
<td>Condition</td>
<td>Cell Type</td>
<td>Phase</td>
<td>Location</td>
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</tr>
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<td>USA</td>
<td>Advanced Cell Technology</td>
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</tr>
</tbody>
</table>

*Table 1: List of embryonic stem cells clinical trials currently collated in the International Clinical Trial Registry Platform (ICTRP) by the World Health Organization. Only clinical trials phase I-III included.*
<table>
<thead>
<tr>
<th>Assay</th>
<th>Intended use</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Karyotyping (G-banding and/or Spectral)</strong>[^23, ^25]</td>
<td>Assess genetic integrity</td>
<td><strong>Advantages</strong></td>
<td>Low genome resolution. Low throughput</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unbiased genome coverage. Can detect balanced translocations and inversions. Cell-level resolution</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Population level resolution</td>
</tr>
<tr>
<td><strong>Comparative Large Scale Expression analysis</strong>[^28, ^31, ^146] (e-Karyotyping)</td>
<td>Assess genetic integrity Assess cell differentiation</td>
<td>High genome resolution. Can probe specific zones. Expression profile and genetic integrity test at the same time.</td>
<td>Indirect test for genetic integrity. Does not detect changes in ploidy. Unable to detect balanced translocations and inversions. Population level resolution</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Population level resolution</td>
</tr>
<tr>
<td><strong>Standard histology and cell microscopy</strong>[^97, ^147]</td>
<td>Assess cell differentiation</td>
<td>Cell-level resolution. Can detect incomplete and immature phenotypes or transformation</td>
<td>Significant experience required. Invasiveness for in vivo and clinical use. Cannot discriminate between host and graft. Low throughput</td>
</tr>
<tr>
<td><strong>Standard molecular biology expression tools</strong> (northern and western blotting, ELISA, 2D protein gels, PCR related techniques)[^25, ^32, ^148]</td>
<td>Assess cell behavior and differentiation</td>
<td>Can detect incomplete and immature phenotypes or transformation Can discriminate between host and graft (depending on technique and application)</td>
<td>Invasiveness for in vivo and clinical use. Population level resolution</td>
</tr>
</tbody>
</table>

| Table 2: Available assays to assess the tumorigenic risk of stem cell therapeutics, describing the main uses of each technique along with advantages and disadvantage |  |
|---|---|---|---|
| **in-situ hybridization and immunolabelling of endogenous transcripts/antigens (including bioluminescence and cell sorting techniques)** | 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** | Assess cell behavior and differentiation | High throughput.  
Unbiased proteome coverage.  
Can detect incomplete or immature phenotypes.  
Can discriminate between host and graft (with labelling) | Significant experience required.  
Sensitivity can be an issue for low abundance proteins.  
Invasiveness for *in vivo* and clinical use. |
| **Standard toxicology studies** | 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.) |
| **3D imaging techniques (MRI, CT, PET scans)** | Assess tumour formation in animals and humans.  
Assess status of graft/device  
Assess host status | Non-invasive.  
Good spatial data.  
Radioactive labelling (PET) can detect specific targets. | Only morphological data (MRI and CT).  
Use of X-rays (CT) and/or radioactive reagents (PET).  
Requires expensive infrastructure. |
<p>| <strong>Photoacoustic imaging</strong> | Assess tumor formation in animals and humans | Non-invasive | Low skin penetration |
| <strong>Bioluminescence imaging</strong> | Assess tumor formation in animals and humans | Non-invasive | Low skin penetration |</p>
<table>
<thead>
<tr>
<th>Strategy</th>
<th>Imaging Modality</th>
<th>Overview</th>
<th>Sensitivity</th>
<th>Spatial resolution</th>
<th>Duration of track</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct Cell Labelling</td>
<td>MRI</td>
<td>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.</td>
<td>- $10^{-3}$-$10^{-5}$ mol/L</td>
<td>- 25-100 μm</td>
<td>- Cell Lifetime (Diluted over time)</td>
<td>- High spatial and temporal resolution</td>
<td>- Combines functional and morphological visualization</td>
</tr>
<tr>
<td></td>
<td>Radionuclide imaging (PET and SPECT)</td>
<td>Ex vivo cellular uptake of radionuclides as a contrast agent (depending on the isotope used the tracking period is used).</td>
<td>- $10^{-10}$-$10^{-12}$ mol/L</td>
<td>- 1-2 mm</td>
<td>- Dependent on isotope half life</td>
<td>- Picomolar sensitivity</td>
<td>- Good tissue penetration</td>
</tr>
<tr>
<td></td>
<td>Optical fluorescence imaging</td>
<td>Cells are labelled ex vivo with quantum dots (QDs) or fluorophores.</td>
<td>- $10^{-9}$-$10^{-12}$ mol/L</td>
<td>- 2-3mm</td>
<td>- 2-14 days (imaging), 8 weeks (QDs: histology)</td>
<td>- High sensitivity</td>
<td>- High photostability (QDs)</td>
</tr>
</tbody>
</table>
Table 3: List of strategies used to directly or indirectly label stem cells in vivo. The table offers an overview of all methods in use for the labelling and the tracking of stem cells, with pros and cons. It should be noted that for the photoacoustic tomography, the technique itself is so new that drawbacks have still to be underlined. The different techniques are further reviewed by James and Gambhir.\textsuperscript{161} QD quantum dots; G/RFP, green or red fluorescent protein.

| Indirect Cell Labelling | Fluorescent Imaging\textsuperscript{161} | Cells are transduced with a gene which encodes for a fluorescent protein (GFP, RFP, etc) | - $10^{-9}$-$10^{-12}$ mol/L  
- 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 |
|---|---|---|---|---|---|
| | Bioluminescence Imaging\textsuperscript{155, 162} | Cells are transduced with a bioluminescent reporter gene | - $10^{-15}$-$10^{-17}$ mol/L  
- 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 |
| | Photoacoustic Tomography\textsuperscript{124, 125, 163, 164} | Cells are transduced with a gene which replies to Photoacoustic waves with waves that are collected to produce a 3D image.  
Gold nanoparticles can also be used | - $10^{-11}$-$10^{-12}$ mol/L (gold nanoparticles)  
- Up to 7 cm  
- Cell lifetime | - Low scattering in tissues  
- Multi-scale high resolution imaging of biological structures  
- 100% sensitivity  
- Background-free detection  
- Speckle-free | - Genetic modification |