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Regenerative medicine: advances from developmental to degenerative diseases

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Abstract

Chronic tissue and organ failure caused by an injury, disease, ageing or congenital defects represents some of the most complex therapeutic challenges and poses a significant financial healthcare burden. Regenerative medicine strategies aim to fulfil the unmet clinical need by restoring the normal tissue function either through stimulating the endogenous tissue repair or by using transplantation strategies to replace the missing or defective cells. Stem cells represent an essential pillar of regenerative medicine efforts as they provide a source of progenitors or differentiated cells for use in cell replacement therapies. Whilst significant leaps have been made in controlling the stem cell fates and differentiating them to cell types of interest, transitioning bespoke cellular products from an academic environment to off-the-shelf clinical treatments brings about a whole new set of challenges which encompass manufacturing, regulatory and funding issues. Notwithstanding the need to resolve such issues before cell replacement therapies can benefit global healthcare, mounting progress in the field has highlighted regenerative medicine as a realistic prospect for treating some of the previously incurable conditions.

Introduction

The ultimate goal of regenerative medicine is to heal diseased or injured tissues and organs either by replacing them or enhancing their regeneration potential *in situ*[1]. Curing the disease using innovative regenerative medicine therapies promises to revolutionise the healthcare of the future. The need for effective regenerative medicine therapies has been intensified by the projections for an increasingly ageing world population and the consequent predicted rise in age-associated degenerative diseases[2]. Against this daunting background, the historical precedent of allogeneic transplantation highlights cell replacement as a conceivable approach to treating degenerative diseases[3]. Nonetheless, the large-scale deployment of such an approach has been limited by the lack of an adequate supply of cells, as the demand for donated tissues and organs by far outweighs the current and future clinical need. The advent of stem cell technologies has had a profound impact on the field of regenerative medicine, providing exciting new perspectives promising to overcome the existing limitations. Indeed, recent years have witnessed tremendous progress towards this goal, with several ongoing clinical trials involving stem cell-derived cells for treatment of degenerative diseases. Here, we start by providing a brief overview of the main types and characteristics of stem cells as the main sources of cells for regenerative medicine therapies. Furthermore, we discuss several examples of the development of stem cell-based therapies for currently incurable diseases caused either by injury (spinal cord injury), degeneration (Parkinson's disease) or developmental anomalies (Hirschprung's disease). Finally, based on the current data and lessons learnt from the past and current stem cell-based clinical studies, we highlight the main outstanding hurdles hampering the translation of stem cell-based cellular therapies into standard clinical practice.

Stem cells as a source of cells for regenerative medicine

Stem cells are broadly defined as cells that have the ability to replenish their own population

(the feature known as self-renewal) and the ability to produce more specialised cell types (differentiation)[4]. These unique features make stem cells an ideal source of cells for regenerative medicine, as they allow production of an unlimited number of cells of a particular type that could be used to replace the missing or diseased cells in the body. Although by definition all stem cells possess the ability to self-renew whilst retaining the ability to differentiate, different types of stem cells can be distinguished based on various criteria. For example, according to their developmental origin, stem cells can be categorised as either adult or embryonic. The adult stem cells are typically found in adult somatic tissues where they maintain tissue homeostasis and are hence also termed tissue-specific stem cells. On the other hand, embryonic stem cells originate from the early embryos[5-7]. The adult and embryonic stem cells also differ in their ability to give rise to differentiated cell types. Adult stem cell differentiation is typically limited to the cell types of the tissue where they reside, a feature known as multipotency. In contrast to this, embryonic stem cells have the ability to produce all of the cell types in the body, and this broad developmental potential of embryonic stem cells is termed pluripotency[5,6].

Multipotent stem cells

Multipotent stem cells support the life-long tissue regeneration and homeostasis due to their ability to produce all of the cell types of their residential tissue or organ. Through seminal work of two Canadian scientists, Till and McCulloch, the hematopoietic stem cell was the first multipotent stem cell identified, and it remains the best characterised stem cell to date[8,9]. Capable of multilineage differentiation to all of the blood lineages, hematopoietic stem cells have to daily replenish billions of cells lost from the hematopoietic system due to the limited life-span of specialised blood cells. Hematopoietic stem cells have also provided a paradigm for cell replacement therapies. Indeed, the transplantation of hematopoietic stem cells has been clinically used since the 1950s as a treatment for blood and bone marrow cancers[10]. The

treatments are based on the ability of transplanted hematopoietic stem cell from a tissue-matched donor to reconstitute all of the blood cells in a patient whose bone marrow has been ablated using irradiation or chemotherapy[11,12].

Another example of a tissue in which a rapid turn-over of specialised cells is underpinned by a self-renewing stem cell population is the intestinal system. The intestine is one of the fastest renewing tissues in the body, with an entire intestinal epithelium being replaced every four to five days, hence warranting a constant production of the differentiated cells[13]. Unlike the hematopoietic stem cells, which are relatively easily accessible and whose functional identity can be shown by a transplantation of a single cell, the identification of stem cells in the gut relied on the lineage tracing analyses[14]. Such analyses revealed the intestinal stem cell at the apex of the intestinal tissue hierarchy, giving rise to differentiated cell types of the gut which carry out their specialised functions[15,16].

In contrast to the rapidly renewing tissues such as blood, gut, and skin, the regenerative capacity of some other tissues, such as the central nervous system is less apparent. Nonetheless, neural stem cells have been identified in the adult central nervous system, albeit mainly limited to restricted regions of the hippocampal dentate gyrus[17] and the subventricular zone of the lateral ventricular wall[18]. Harnessing the therapeutic potential of the neural stem cells could be possible either through stimulating their regenerative capacity *in vivo*, or purifying them and expanding *in vitro* prior to the therapeutic applications[19]. However, given the difficulties in isolating neural stem cells from *in vivo* sources, a promising alternative supply of neural stem cells are human pluripotent stem cells, which appear to have the ability to generate large numbers of neural stem cells that can be patterned to various sub-types useful for regenerative medicine[20].

Pluripotent stem cells

Defined by the ability to self-renew and give rise to cells from all three embryonic germ layers (ectoderm, mesoderm, and endoderm), the two types of pluripotent stem cells with likely clinical applications are human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). HESCs were first derived in 1998, almost twenty years after the establishment of mouse embryonic stem cell lines[7]. The publication of the seminal paper describing the process of derivation of hESCs from donated surplus IVF blastocysts spurred a flurry of interest into stem cell research. The ability of hESCs to differentiate into somatic cell types *in vitro* was quickly recognised as an enormous opportunity for basic research, disease modelling and, most importantly, as a long-awaited source of cells for regenerative medicine. In another remarkable breakthrough in the field, Yamanaka and colleagues generated human induced pluripotent stem cells (hiPSCs) by reprogramming somatic cells using only four transcription factors[21]. In addition to alleviating some of the ethical issues associated with the derivation of hESCs, reprogramming of somatic cells allows derivation of patient-specific pluripotent cells. This, in turn, provides a platform for personalised approach to medicine, be it for disease modelling and drug discovery or for the production of patient-specific (and hence immuno-compatible) cells for cell replacement therapy (**Figure 1**).

A number of intracellular and cell-surface markers are associated with the undifferentiated state of hPSCs and hence used to identify undifferentiated cells in culture. These include core pluripotency transcription factors POU5F1 (OCT4) and NANOG, and cell surface antigens such as TRA-1-60, TRA-1-81, SSEA3, and SSEA4[22]. However, it should be noted that whilst the aforementioned markers can be used to assess hPSC phenotypes, the true definition of hPSCs is based on their functional features of self-renewal and differentiation. Therefore, assessing whether a cell is a true stem cell should ultimately test its functional attributes. This is one of the major difficulties when assessing hPSCs, since their true developmental potency can only be demonstrated by placing cells in an environment where they

can differentiate to cells from all three parent lineages that go on to generate the complete embryo. Equivalent experiments are performed with murine PSCs, whereby the cells are injected into a mouse blastocyst, followed by embryo transfer into a pseudopregnant foster female. If the injected PSCs are truly pluripotent, they will contribute to all the cell lineages in the chimeric mouse, including the germ line. Obviously, ethical principles do not allow this type of functional assessment of hPSCs, and alternative *in vitro* and *in vivo* assays are used as surrogate tests for assessing functional aspects of hPSCs. In particular, the teratoma assay has been considered a gold standard test of pluripotency[23]. The assay entails injecting hPSCs into an ectopic site of an immunodeficient mouse. In such an *in vivo* environment, hPSCs tend to form complex tumours (teratomas), containing differentiated cells and haphazardly organized tissues. Importantly, the cells and rudimentary tissues in teratomas are of ectodermal, mesodermal and endodermal origin, and the presence of cells from all three embryonic germ layers has been considered as evidence of pluripotency of injected hPSCs[24].

Differentiation of human pluripotent stem cells

Differentiation of stem cells to desired specialised cell types is an essential prerequisite to utilising these remarkable cells for therapeutic applications. Nonetheless, although in theory hPSCs can make any cell type in the body, deciphering instructive cues that drive these unspecialised cells to a fully functioning mature cell type of choice has proven an arduous task. Early efforts of finding the appropriate differentiation protocols for hPSCs have been focused on the production of just a handful of cell types out of over two hundred possible differentiated types that build the human body. The cell types in question were deemed to have the greatest therapeutic significance, including pancreatic beta cells, hepatocytes, cardiomyocytes, and neurons[25]. These early efforts of producing specialised cell types from hPSCs were plagued by issues concerning differentiation efficiency, robustness, and reproducibility. It is worth noting

that the early methods for culturing hPSCs entailed growing them on a layer of mitotically inactivated mouse embryonic fibroblasts in a media that included fetal bovine serum. Not surprisingly, such a chemically undefined culture system suffered from batch-to-batch variability and a consequent lack of reproducibility. Some of the robustness and reproducibility issues also appeared to be due to seemingly differing propensities of hPSC lines for differentiation to specific lineages[26]. In the years that followed, meticulous studies contributed to vastly improved differentiation protocols, directing hPSC differentiation to a number of cell types of interest. Although arguably each of the differentiation protocols developed had specific intricacies, some of the common denominators started to emerge as key principles that can be applied to instructing hPSC differentiation. In particular, the majority of successful protocols for hPSC differentiation in a monolayer are based on mimicking cues that the cells experience during normal embryonic development[25]. Admittedly, not much is known about the very early stages of human embryonic development *in vivo*, due to the inaccessibility of the early embryo and the ethical concerns with performing human developmental studies. Nonetheless, very informative studies on the development of other mammalian species and the differentiation studies of the mouse embryonic stem cells have provided the crucial insight into the signalling prompts that hPSCs may experience during development. In line with mimicking the developmental processes, the successful differentiation of hPSCs typically requires stepwise protocols, whereby each stage of differentiation is carefully instructed with specific signalling cues before the ensuing progenitor cells are exposed to a new set of signals. A seminal study by Wichterle *et al.*[27] demonstrated this paradigm by differentiating hPSCs to motor neurons through sequential manipulation of signalling pathways that underpin motor neuron specification during embryo development. This concept was subsequently applied to generate numerous cell types from hPSCs, including various neuronal subtypes (spinal motor, cortical, DA and GABA neurons), cardiomyocytes, hepatocytes and β-cells (summarised in [28]).

Progress in the development of hPSC-based cell replacement therapies

The establishment of protocols for hPSC differentiation to various differentiated cell types has spurred progress of hPSC-based cell replacement therapies towards clinical trials. Here we give a brief overview of the progress in the trials for spinal cord injury and Parkinson's disease. Several other ongoing clinical trials are examining the safety/efficacy of cell replacement therapies for the treatment of chronic conditions such as retinal degeneration, heart failure and diabetes (reviewed in [29]). In addition, driven by immense clinical need and the ability to obtain appropriate cell types, further clinical studies may be on the horizon. We highlight a developmental disorder, Hirschprung's disease, as a condition potentially amenable to treatment by cell replacement therapy.

Regenerative medicine approach to treatment of spinal cord injury

Spinal cord injury is one of the key target injuries for a regenerative medicinal approach. There are more than 10,000 new cases per year in the USA, with long-term repercussions for sufferers requiring constant care resulting with an estimated cost of $4 billion annually (reviewed in[30]). Permanent paralysis and loss of sensation upon traumatic spinal cord injury is caused by the death of neurons and glia cells. In some cases, a key issue arises from demyelination of otherwise intact axons, leading to the loss of function and degeneration of neurons[31]. In such cases, a potential approach for treating spinal cord injury could entail transplanting the patients with cells capable of remyelinating spinal cord neurons in order to prevent their degeneration. Such an approach was tested in animal models of spinal cord injury, whereby animals were transplanted with progenitor cells capable of differentiating into oligodendrocytes *in vivo*. Cell types that have been tested as a source of cells for generating oligodendrocyte progenitors prior to transplantation include hES cells[32,33], neural stem cells[34] and hiPS cells[35]. Given the ability of

hPSCs to give rise to an unlimited number of cells *in vitro*, they were considered a particularly promising source of cells for therapeutic applications[32,33]. Recovery of motor function in animal models of spinal cord injury provided an impetus for clinical trials to test safety and efficacy of hESC-derived oligodendrocytes for the treatment of spinal cord injury[36]. Clinical trials were commenced by the Geron corporation in 2010, with the Phase I of the trial designed to test the safety of the product through dose escalation. The starting dose was two million cells injected into the spinal cord of each patient. For spinal cord injury, this represents a relatively low dose as calculations based on the equivalent experiments in rat models indicate that 20 million cells would need to be transplanted for any rescue of function. In line with that, no major improvements were noted in the patients' motor function in the safety trial. Minor adverse events were reported when patients were checked one week to one year post-transplantation, but there was no evidence of serious adverse events, tumours or rejection of the transplanted cells. Although this clinical trial was initially met with optimism, it was terminated after two years for commercial reasons[37]. Recently, Geron's oligodendrocyte differentiation protocol was acquired by Asterias Biotherapeutics, who are in the process of recruiting for a follow-up safety trial (http://www.scistar-study.com/).

Regenerative medicine approach to treatment of Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative condition with the characteristic clinical features of tremor, rigidity, and slowness of movement, together with a range of non-motor features. It affects 1-2% of the population over the age of 65[38] and has a significant burden of disease. There is currently no treatment that alters the course of the disease and 60% of patients progress to severe disability or death within eight years of diagnosis[39]. The pathological hallmark of the disease is the loss of a specific subtype of dopaminergic neurons from the substantia nigra within the midbrain. The focal loss of this relatively rare population of cells

makes the disease a prime target for cell replacement therapy.

Efforts to identify a viable source of dopaminergic cells for transplantation to the PD-affected brain began in the 1970s. Grafts derived from fetal ventral mesencephalon (fVM) showed the most promise with evidence of successful engraftment into the host brain, the ability to release dopamine and to ameliorate motor deficits in experimental animals[40]. The outcome of human transplantation of fVM tissue has met with variable results but there it is clear that in some cases it provides an effective and durable therapy with some patients able to remain off pharmacotherapy for over 15 years after transplantation[41]. Whilst this work provides a proof of concept that cell replacement strategies can be a successful treatment for Parkinson's Disease, the ethical and availability issues associated with fetal tissue preclude this from being a viable therapy outside of research studies.

Advances in stem cell and neural differentiation techniques now raise the prospect of being able to replicate the positive clinical outcomes using pluripotent stem-cell derived graft material. A major achievement in the field was the development of protocols, which can generate high yields of the specific A9 dopaminergic neuron subtype that is affected in the disease[42]. These protocols have now been adapted to clinical grade reagents and culture conditions and preclinical work has demonstrated these cells to be safe and have a similar efficacy to fetal-derived cells when transplanted in animal models[43]. It is expected clinical trials will begin over the next few years at a number of centres around the world[44].

For these initial trials, an allogeneic approach using a single hESC or hiPSC source cell line has been favoured. The relatively immunologically privileged status of the brain means that the lifelong immunosuppression may not necessarily be essential in CNS transplantation. In patients who have been transplanted with fetal cells from an allogenic source, it has been demonstrated that a finite period of immunosuppression following transplantation (ranging from 12 months to 5 years) is sufficient for tolerance to the cells to be induced, with evidence that the

grafts can then continue to survive for decades in the absence of immunosuppression[41,45]. Despite this, two groups have been working towards an autologous hiPSC-derived cell therapy for Parkinson's disease[46,47], although one of these groups appears to have shifted focus to allogeneic haplobanked hiPSCs. One alternative approach that has already reached clinical trial has involved the use of parthenogenic stem cells. This is the first clinical trial to be approved for a stem-cell derived therapy in Parkinson's disease, with the first patient treated in 2016. There are, however, concerns that the differentiated cell type used in this trial may not be optimal and that the supporting pre-clinical data for this approach was limited[48]. It will be several years before it will be known if cell replacement therapies can provide an effective and viable therapy for Parkinson's disease. Not only with this potentially provide a first example of the use of stem cell-based therapy for a neurodegenerative disease, but will also provide information about the relative merits the different source material and differentiation strategies being employed by different groups to produce their cell transplantation product.

Regenerative medicine approach to treatment of Hirschprung's disease

Hirschsprung's disease is a congenital disorder with an incidence of 1 in 5000 live births (reviewed in[49]). The patients are born with a segment of gut that is not innervated by the enteric nervous system, resulting in the constricted colon and the inability of patients to defecate[50]. Unless there is surgical intervention to remove non-innervated part of the gut within 24 hours of birth, Hirschsprung's disease is fatal[51]. Given that the disease is underpinned by an absence of a particular cell type – enteric neurons - cell replacement therapies have been thought to be the ideal treatment for Hirschsprung's disease[52]. The key regenerative medicine approaches for Hirschsprung's disease would involve deriving the correct precursors for the enteric nervous system, transplanting them *en masse* and allowing them to migrate, differentiate and integrate with the gut, thus allowing for the rescue of peristalsis and relaxation of the constricted gut[53].

Due to the sheer length of the gut, it is essential for sufficient numbers of cells to be generated for any regenerative therapy, which has led to significant work into conditions that will allow for expansion of enteric progenitors prior to differentiation into enteric neurons. Considerable promise has been demonstrated with studies from mouse gut stem cells, where both fetal and adult stem cell populations have been isolated, expanded and shown to form neural and glial derivatives after transplantation *in vivo*[54,55]. The ability to form neurospheres that generate neurons and glia is not limited to gut stem cells. Central nervous system-derived stem cells have also been touted as a source of cells for enteric nervous system transplantation[53]. In addition, there has been significant progress in generating enteric neurons from hPSCs[56]. Transplantation of hPSC-derived enteric neuron precursors into a mouse model of Hirschsprung's disease (Ednrb-/-) led to the migration of cells along the gut and rescued the mutant mice from dying[56]. HPSC-derived enteric neurons can also innervate hPSC-derived gut organoids, which represent the three-dimensional models of the gut tissue comprising various cell types present in the gut epithelia as well as the smooth muscle that surrounds it[57]. Combining the enteric neural progenitors with gut organoids led to the formation of enteric ganglia and innervation of the smooth muscle, thus allowing the control of peristalsis[57].

Whilst such preclinical studies provide proof of concept for cellular replacement approaches for the treatment of Hirschsprung's disease, more work is warranted to specifically assess long-term safety and functionality of any transplanted cells. The presence of immune cells in the gut is an added complication to this therapy that may not present an issue in the treatment of some other diseases, such as Parkinson's. In addition, the number of cells required for treatment of Hirschsprung's disease is a major hurdle to be overcome. Indeed, due to the length of the gut, it is anticipated that transplantation of vast amount of cells will be required to sufficiently reinnervate aganglionic areas. Apart from the issues of producing the large numbers of cells, this will also have implications for the method of transplantation. For example, an

injection of cells, which appears a choice delivery of cells for Parkinson's disease, may not be the best method for Hirschsprung's disease. Current preclinical methods are utilising neurospheres or cells encased in extracellular matrix, but these methods have not been optimised as yet to achieve the best functional rescue. Potentially the best method will be a combination of hPSC-derived enteric neural crest cells in a bioengineered device, which can assist in cell grafting and reducing immunogenicity[58].

Lessons gleaned from past and current (pre)clinical studies

As we await results from current clinical trials on the safety and efficacy of hPSC-derived cellular products in regenerative medicine therapies, it is appropriate that we take stock of key that have plagued clinical translation thus far, with a view of informing future developments in the field.

The challenge of producing specific mature cell types

The ability to control and direct differentiation of hPSCs to desired functional cell types is an essential prerequisite for regenerative medicine efforts. Significant progress has been made towards establishing chemically-defined protocols for hPSC differentiation to a variety of cells types, including cardiomyocytes[59], hindbrain and spinal cord neural stem cells[60], epicardial cells[61], and vascular smooth muscle subtypes[62]. However, many of the protocols yield the differentiated cells that exhibit a relatively immature phenotype. For example, phenotypes of hPSC-derived cardiomyocytes reflect structural, molecular and electrophysiology phenotypes of fetal, rather than fully mature adult cardiomyocytes[63,64]. Similarly, phenotypic and functional features of hPSC-derived hepatocytes[65] and β cells also appear to align more closely with their fetal rather than the adult counterparts[66]. Although fetal-like cells will undoubtedly prove useful in developmental studies and some aspects of disease modelling, cell replacement therapies

necessitate the production of cells capable of generating fully functional adult cell types when transplanted. Several strategies are being explored to promote maturity of the hPSC-derived fetal-like cells, encompassing both *in vitro* and *in vivo* approaches (reviewed in[67]).

A further issue hindering the formulation of robust differentiation protocols is the inter-line variability of hPSCs in the propensity to differentiate into particular cell types. The differentiation bias of hPSCs was revealed in studies that examined the efficiency of the same differentiation protocol on a variety of different hESC or hiPSC lines in parallel[26,68,69]. The conclusions drawn from such studies indicated that some lines readily differentiate to cell types of interest, whilst others yield a very low efficiency of desired differentiation. A low efficiency of differentiation may result in the exclusion of a cell line from use, a practical solution that is particularly undesirable when small numbers of patient-specific or haplotype-matched hPSC lines are available. Alternatively, the differences in the differentiation propensity may warrant optimisation of the differentiation protocols for each hPSC line, which can be time consuming and expensive. Hence, future research is needed to unravel the factors that underlie the observed differences in differentiation capacity of hPSC lines. An important step towards this goal was made in a recent study that examined the molecular features of hiPSC lines that exhibited high and low propensity to differentiate to hematopoietic stem cells[69]. Based on this study, the analysis of epigenetic landscape of hPSCs appears to be a promising way forward for predicting the differentiation potential of hPSC lines and selecting the optimal lines for downstream applications.

The outcome of the transplantation: the importance of the supportive niche and absence of immune reaction

Obtaining appropriate cell types for transplantation *in vivo* represents only a part of the challenge in restoring normal tissue function, with another major hurdle being the survival and

functionality of the transplanted cells. Indeed, it appears that less than 1-3% of total transplanted cells survive initial transplantation *in vivo*[70,71]. One of the major reasons behind a failure of cells to thrive upon transplantation is thought to be the absence of supportive environment or a niche. Diseased, aged or injured tissues may not provide sufficient levels of oxygen or present the signals necessary for cell survival. On the other hand, the engraftment of cells even in healthy adult tissues is generally limited, possibly due to the lack of available niches for the transplanted cells. In that respect, it is telling that successful bone marrow transplants require ablation of the bone marrow to kill off host cells occupying the niche before repopulation with transplanted cells. Nonetheless, the approach of killing off host cells occupying the niche is clearly not a feasible approach for many diseases, including Parkinson's. For some diseases, it has been speculated that a transplantation of stromal cell types might aid in rescuing tissue function through helping to generate a supportive niche for transplanted cells. For example, oligodendrocyte precursors for spinal cord injury have been demonstrated to release trophic factors after transplantation, which show positive effects on spinal cord neurons *in vitro*[72].

In addition to the lack of a supportive environment, the death of transplanted cells can also be mediated by the immune reaction of the host. Regenerative medicine encompasses a wide range of potential therapeutic strategies, from the transplantation of allogeneic replacement tissue generated *in vitro* to the use reprogrammed cells transplanted autologously, and potentially the *in vivo* transdifferentiation of supportive cells to perform the function of a disease cell type. The ability to avoid the use of immunotherapy is one of the reasons put forward in favour of autologous forms of treatment. However, many of the therapies that are closest to, or currently in, clinical trials are those that involve allogeneic grafts generated from hPSCs of a single cell line. In these circumstances, it is necessary to achieve immune tolerance of the graft, either through the use of immunosuppressive agents or other means. This may not necessarily be at the same high doses required for solid-organ transplantation and may not

necessarily be life-long. In circumstances where the cells are transplanted to an immunologically privileged site such as the brain or the anterior chamber of the eye, a finite period of immunosuppression may be sufficient. An alternative to the use of immunosuppression may be the induction of tolerance. Recent work has indicated this may be possible to generate long-term tolerance to stem cell-derived grafts by using CD4/CD8 coreceptor and costimulation (CD40L) blockade with monoclonal antibodies given at the time of transplantation[73]. A further alternative strategy in development is the use of genetic engineering of the HLA locus to create a universal cell that is able to evade the alloimmune response[74]. With these developments, the issue of immunosuppression is not necessarily an overriding consideration and it will be of interest to see whether the advances in technologies supporting the efficient production of clinical-grade, regulator-approved, autologous iPSC lines outpaces the advances in strategies to obviate the need for immunosuppression in the allogeneic setting.

Safety of the hPSC-derived cellular products

Safety of the hPSC-derived cell replacement therapies is at the forefront of concerns in the regenerative medicine field, with a particularly critical issue being the potential tumorigenicity of transplanted cells. This issue stems partly from the fact that the undifferentiated hPSCs have the ability to form teratomas when placed into ectopic sites in immunocompromised mice[24]. In this context, it is important to note that cell replacement therapies are based on using derivatives of hPSCs and not the undifferentiated cells *per se*. Thus, strategies for minimising the risk of remnant undifferentiated hPSCs following the differentiation, for example by sorting the cell populations or by eliminating undifferentiated cells through chemical treatment, should be effective in minimising the risk of teratomas. A similar strategy could be used for eliminating other unwanted cell types that may be present in a cellular preparation at the end of the

differentiation protocol. It has been speculated that 'contaminating' cell types could also present a safety issue in some situations, particularly if they are transplanted to a tissue or a niche in which they do not typically reside[75]. Whilst efficient purifying and monitoring methods should alleviate the tumorigenic risk of undifferentiated hPSCs or contaminating cell types, more challenging to tackle is the potential tumorigenicity of hPSC-derived differentiated derivatives. The observation that hPSCs acquire genetic aberrations during culture[76] has raised concerns that some of the genetic changes may go undetected at both genotype and phenotype levels in hPSCs[77], but may confer malignant properties to differentiated derivatives when placed in an *in vivo* environment. Such a concern precipitated a halt of a clinical trial for age-related macular degeneration in Japan when patient-derived hiPSCs were found to contain several genetic changes that were not present in the somatic cells used for reprogramming[78]. In light of these findings, the scientists involved in the trial decided to err on the side of caution, thus suspending the trial and changing their strategy to using haplotype-matched donor cells[78]. The use of partially matched donor cells will allow extensive genetic characterisation of a large batch of cells which should be time- and cost-efficient compared to characterising individual patient-specific hiPSC lines. Nonetheless, the challenge remains to determine which genetic changes represent a potential safety issue for cellular replacement and which are merely innocuous genetic events. In addition to potential tumorigenicity, another risk factor for cellular therapies is the presence of adventitious agents and disease transmission from transplanted cells. Traditional sterilization is not applicable in case of cellular products, hence mitigating the risk of viral and bacterial transmission includes both testing for adventitious agents and manufacturing in compliance with Good Manufacturing Practice (GMP).

Regulatory landscape

The challenges faced by developers of regenerative therapies do not end with the successful

generation of a target cell type and the demonstration of efficacy in preclinical studies. To proceed to a Phase I clinical trial, approval for use of the therapy in humans is required from the relevant national or international regulatory bodies such as the US FDA or European EMA. In general terms, to satisfy regulatory requirements a cell therapy must have a production process that is well-controlled, reproducible and capable of generating a cell product within well-defined specifications. All reagents and processes must comply with clinical-grade Good Manufacturing Practices (GMP). In addition, the safety of the cell product must be demonstrated using data combined from animal studies, cell karyotyping or other genetic analyses, as well as testing using standard assays for sterility and adventitious agents. For cell products that have been derived from hPSCs, it is critical that the cell product is evaluated for tumorigenicity through the use of animal transplantation and biodistribution studies as well as flow cytometry or other single cell analyses to exclude the possibility of contamination of the final cell product with potentially oncogenic pluripotent cells.

Demonstrating safety and meeting regulatory requirements for a therapy in which the therapeutic agent is a population of living cells is, unsurprisingly, more difficult than a conventional pharmacological drug. The inherent heterogeneity of hPSC cultures and variability of differentiation procedures is a fundamental issue. Even in well-established clinical grade protocols, it remains difficult to completely eliminate all run-to-run variation. For allogeneic stem cell therapies, another challenge is the identification of a suitable source stem cell line. There are many requirements that need to be considered including whether the cell line was generated in clinical GMP conditions, whether the donor consented to use of the donated material for use in commercial product, the country of origin in relation to prion disease and other infectious risks, and whether the cell line carries any potentially harmful mutations. For example, it has recently been shown that a significant proportion of the global hESC lines carry mutations in *TP53* or other potentially oncogenic loci (Merkle and Eggan, webinar

https://www.stemcell.com/pluripotent-lounge). Whilst the final safety testing, cell production and quality control assays are performed in certified GMP and GLP laboratories, the development of these methods is performed in a standard research environment, usually in an iterative process until the necessary parameters are met to justify moving to the next phase with much higher associated costs (**Figure 2**).

Regenerative medicine: the feasibility of personalised cell products

The advent of techniques for generating induced pluripotent stem cells has given rise to much hope about the prospects for personalized cell therapies that are generated specifically for each individual patient and transplanted autologously, circumventing the need for immunosuppression. It is now technically possible to achieve this, but stem cell lines need to be generated for each individual patient, and these each need to individually pass through extensive safety testing and regulatory requirements before proceeding to transplantation. It is estimated that safety testing alone cost US$500,000[79], with the total cost per patient estimated to be US$1,000,000. This was the approach attempted by a clinical trial based at the RIKEN Institute in Japan for the treatment of age-related macular degeneration, which was halted due to mutations detected in hiPSCs of one of the patients[80]. The suspension of the trial brought sharply into focus the fact that, at the present time, the logistical and financial challenges of developing an autologous hPSC-based therapy are very significant. This does not necessarily mean that the barriers will remain as high. With technical advances and improving understanding, personalised cell therapies may become a more feasible option in the future, with many research groups focussed on this as an objective.

Concluding remarks

Regenerative medicine is on the cusp of transforming healthcare by delivering curative

treatments for many life-threatening or debilitating diseases. The major driving force behind the dynamic evolution of regenerative medicine has been the remarkable progress in the field of stem cells and related technologies. If we look forward, it seems that the rate-limiting step for the development of cell replacement therapies will not be the production of desired cell types, but rather, translation of the developments from an academic into the clinical setting. Unlike drug discovery, which has a well-established manufacturing and regulatory trajectory, when it comes to hPSC-derived cell replacement therapies, we are navigating unchartered waters, full of unforeseen scientific, manufacturing, regulatory and funding complexities. Nonetheless, the preliminary results of the safety studies are encouraging and the prospects for the hPSC-derived cellular therapies appear positive. As highlighted in this review, several hurdles are still hampering the translation but they are surmountable. The continuation of efforts to develop a sound translational framework will undoubtedly help regenerative medicine to deliver its full potential and become an important part of modern healthcare.

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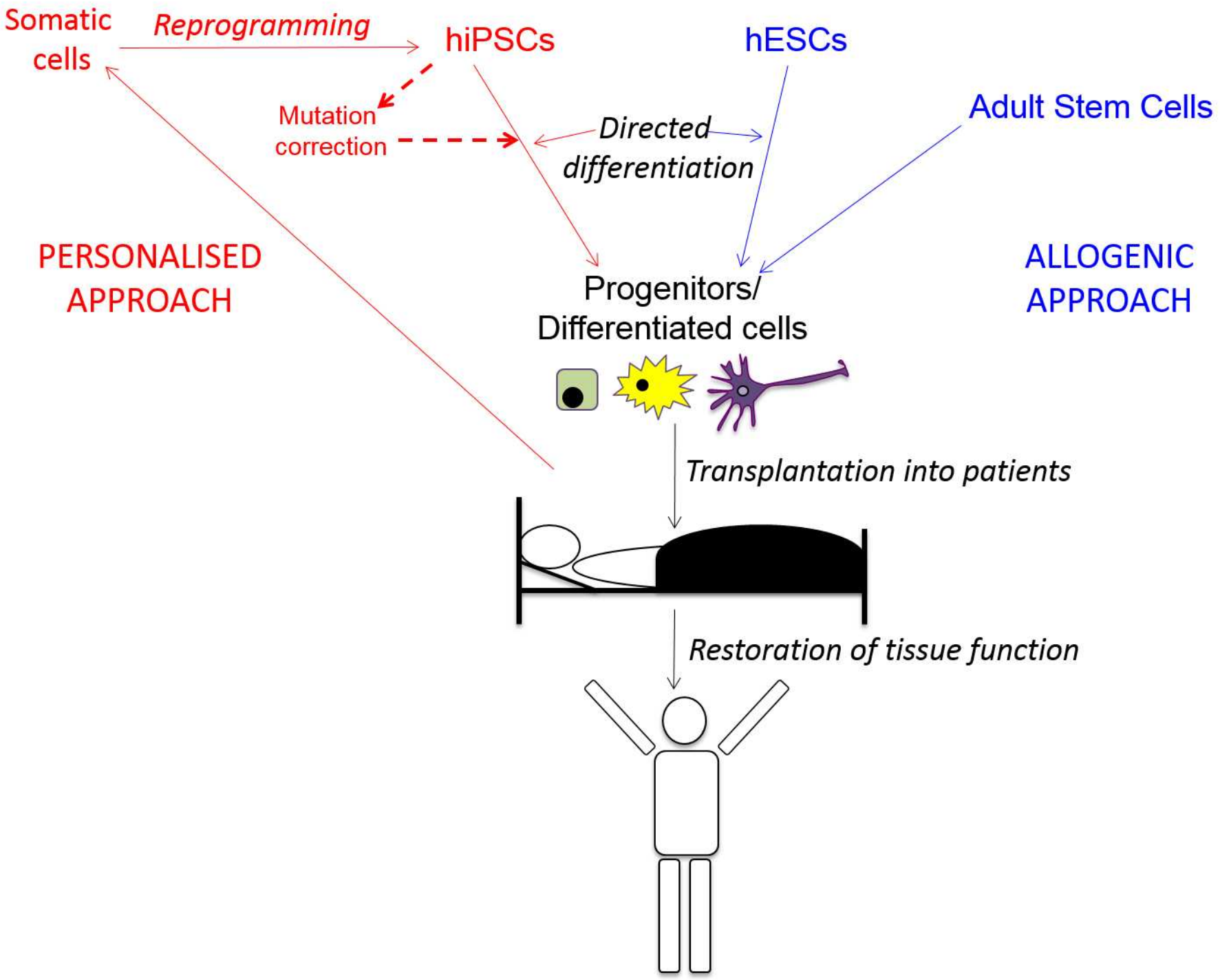
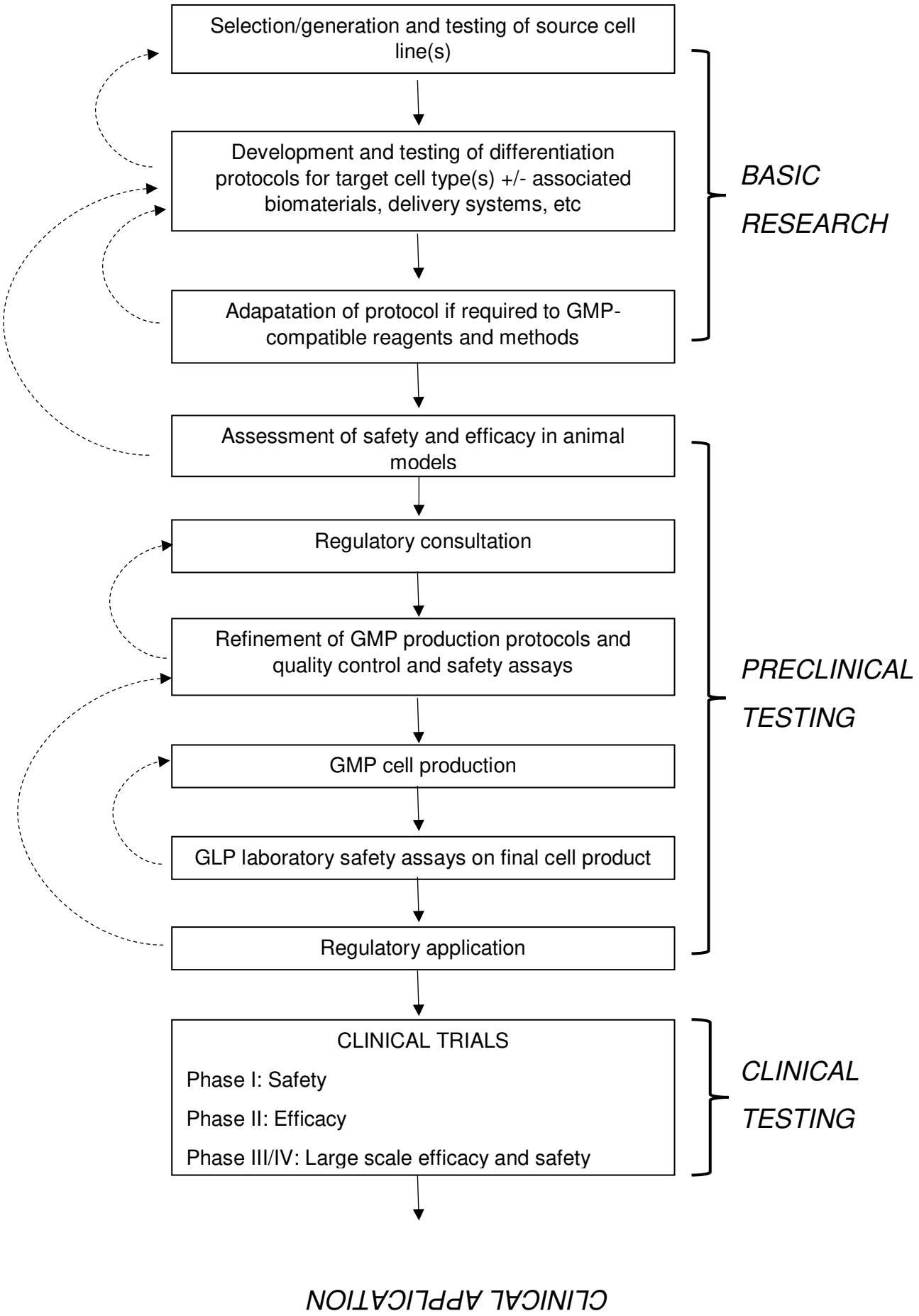
Figure 1. The cell replacement therapy paradigm. Two main approaches include allogeneic approach using adult stem cells or human embryonic stem cells (hESCs) (*right*), and personalised approach, which utilises patient-derived human induced pluripotent stem cells (hiPSCs) (*left*). In either approach, stem cells are directed to differentiate to desired cell types prior to transplantation into patients. In the case of genetic disorders, it may be necessary to correct the disease-causing mutation in hiPSCs by genetic engineering in order to generate functional patient-specific differentiated cells (mutation correction).

Figure 2. Flow chart of typical steps in development of cell therapy products. Starting with basic biology experiments, which encompass development of differentiation protocols and *in vitro* characterisation of differentiated cells, the process is continued by testing the safety and functionality of derived cells in animal models *in vivo*. Positive outcome of pre-clinical testing provides a base for clinical trials in humans. The dashed lines represent iterative loops that may be necessary to optimize the final product.


Somatic cells
Reprogramming
hiPSCs
hESCs
Adult Stem Cells
Mutation correction
Directed differentiation
PERSONALISED APPROACH
ALLOGENIC APPROACH
Progenitors/ Differentiated cells
Transplantation into patients
Restoration of tissue function


Selection/generation and testing of source cell line(s)
Development and testing of differentiation protocols for target cell type(s) +/- associated biomaterials, delivery systems, etc
Adapatation of protocol if required to GMP-compatible reagents and methods
BASIC RESEARCH
Assessment of safety and efficacy in animal models
Regulatory consultation
Refinement of GMP production protocols and quality control and safety assays
GMP cell production
GLP laboratory safety assays on final cell product
Regulatory application
PRECLINICAL TESTING
CLINICAL TRIALS
Phase I: Safety
Phase II: Efficacy
Phase III/IV: Large scale efficacy and safety
CLINICAL TESTING
CLINICAL APPLICATION