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Embryonic stem cell transplantation: potential applicability in cell replacement therapy and regenerative medicine

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1. ABSTRACT

Embryonic stem cells are derived from the inner cell mass of the trophoblast, and have the ability to differentiate into all the tissues of the fetus. As such, their potential in cell replacement therapy and regenerative medicine has been widely acknowledged. Useful cell types such as neurons, cardiomyocytes, hepatocytes, pancreatic beta cells, and blood cells have all been successfully derived in the laboratory. Furthermore, embryonic stem cells may be utilized in novel immunomodulatory applications, such as hematopoietic chimerism strategies aimed at inducing tolerance to donor organ allografts. Unfortunately, progress in embryonic stem cell therapeutics continues to be hindered by haphazard differentiation and tumorigenesis; and the immune response to an embryonic stem cell-derived tissue graft is still an open question. This review summarizes the current state of embryonic stem cell research in regards to transplantation, highlighting the successes to date and the future obstacles yet to be overcome. Although embryonic stem cells are still far from their debut in the clinic, continued scientific advances engender optimism that they will eventually play an important role in cell replacement therapy and regenerative medicine.

2. STEM CELLS: AN INTRODUCTION

A milestone in developmental biology was achieved in 1981 with the successful isolation of mouse embryonic stem (ES) cells (1). When this achievement was followed 17 years later with the derivation of pluripotent human ES cells (2), both scientists and clinicians envisioned a fundamental revolution in the field of regenerative medicine and transplantation. ES cells are derived from the inner cell mass of the blastocyst, and have the potential to generate all the tissues of the body. In addition to their plasticity, these unique cells are able to undergo a process termed "self-renewal", allowing them to be passaged indefinitely *in vitro* under non-differentiating culture conditions. Mouse ES cells are dependent on leukemia inhibitory factor (LIF) to maintain an undifferentiated state (3, 4). When implanted back into the mouse embryo, they are able to contribute to all the tissue types of the fetus, thus proving their pluripotentiality (5). Human ES cells have also shown this spontaneous ability to generate all three embryonic germ layers (6).

2.1. Adult vs embryonic

Stem cells may be divided broadly into two classes: adult and embryonic. Adult stem (AS) cells are

responsible for maintaining cell turnover homeostasis and organ-specific regeneration, whereas ES cells play a central role in embryogenesis and the formation of fetal tissues. Despite some similar characteristics, these two cell types exhibit important differences which have significant implications for their potential use as a cell replacement therapy.

Previously thought to have limited plasticity, AS cells recently enjoyed renewed popularity due to reports of their so-called transdifferentiation capacity; but some studies suggest that these findings are perhaps artifactual. During the early stages of stem cell research, the accepted dogma had been that AS cells, such as hematopoietic stem cells (HSCs), were capable of generating a number of different cell types (in the case of HSCs, all the cells of the blood and immune system), but were unable to form completely unrelated tissues (such as heart or liver). Hence, their plasticity was termed multipotent, as compared to the pluripotency of ES cells or the totipotency of the fertilized egg. Around the turn of the millenium, however, a plethora of evidence surfaced supporting the revolutionary concept of AS cell transdifferentiation. HSCs were reported to have transdifferentiated into neurons (7), muscle (8), and hepatocytes (9). Conversely, muscle satellite cells seemed capable of forming blood (10), and neural stem cells had developed into blood (11) and muscle (12). These early reports of AS cell transdifferentiation achieved considerable acclaim until criticisms arose that cast significant doubt upon their validity. For example, the issue of contaminating populations of myogenic precursors (13) seemed to refute reports of 'blood into muscle', whereas artifacts of *in vitro* culture cast a pall over claims of neural stem cell transdifferentiation (14). These criticisms led to the establishment of three vital criteria for the determination of stem cell transdifferentiation: identification of stem cells should not be based solely on phenotype, use of individual stem cells would avoid contamination from alternative cell types, and *in vitro* culture of stem cells should be avoided (15). In light of these criteria, an astonishing result was published in the journal Cell in 2001 which seemed finally to prove the transdifferentiation capability of HSCs (16). The investigators used a single HSC that was not cultured *in vitro* and was isolated on functional rather than phenotypic criteria to reconstitute an irradiated animal. The results showed that the single donor HSC contributed to recipient populations of endogenous stem cells such as type II pneumocytes, as well as to epithelial tissues of all organs except the kidney (16). Finally, the rigorous criteria to prove true transdifferentiation had apparently been met. Unfortunately, even this study has since been proven to be almost certainly artifactual due to the discovery of a further property of stem cell behavior: cell fusion (17, 18). Recently, the epithelial-mesenchymal cell transition hypothesis has been postulated as a link between AS and ES cells, bringing a new perspective to the possibility of AS cell transdifferentiation (19). In sum total, the evidence to date tends to suggest that AS cells cannot unequivocably transdifferentiate into multiple unrelated tissue types. Additionally, AS cells are often inaccessible or difficult to obtain in large numbers. Therefore, even if AS cells were able to transdifferentiate in some situations, their potential for cell replacement therapy is perhaps limited, and ES cells may prove to be a better alternative.

2.2. Fetal stem cells are intermediate between embryonic and adult

Although the broad categories of adult and embryonic stem cells offer a convenient dichotomy in discussing the potential for therapeutic stem cell applications, fetal stem (FS) cells can be thought of as a an "in between" category possessing greater plasticity than AS cells while unfettered by the ethical controversies surrounding ES cells. FS cells can be derived from fetal blood, liver, bone marrow, amniotic fluid, and placenta, and are rich in a population of stem cells which proliferate more rapidly and exhibit greater multipotentiality than their adult counterparts (20). When transplanted into fetal mouse recipients, fetal liver-derived HSC showed a markedly greater ability to engraft when compared to adult bone marrowed (21). Furthermore, Holyoake *et al.* also compared fetal liver-derived HSC with their adult bone marrow counterparts, and reported that the former were able to produce much larger numbers and types of progeny after transplantation in mice (22). There have even been reports of fetal mesenchymal stem cells (MSC) transdifferentiating into muscle cells and nerve cells, as opposed to the normal osteogenic, chondrogenic, and adipogenic trilineage restriction of adult MSC (23, 24). Hence, FS cells may enjoy certain advantages over adult stem cells in specific applications, such as HSC transplantation. However, their ability to robustly transdifferentiate is far from proven, falling under the same scrutiny and criticisms levelled at AS cell transdifferentiation claims (24, 25). Additionally, the absolute numbers of FS cells that can be generated is even less than that of AS cells, further hindering their widescale applicability to regenerative medicine. Clearly, adult and various fetally-derived stem cells may have useful therapeutic applications and further investigation of these cell types is worthwhile. The unlimited capacity for self-renewal and the pluripotentiality of ES cells, however, remains unmatched. These two factors continue to increase the relative potential of ES cells in cell replacement and regenerative therapies.

2.3. The promise of embryonic stem cells

As a result of two decades of intense research, ES cells have been subject to rigorous definition at both the cellular marker and functional level. Phenotypically, ES cells express the normal surface markers SSEA-4, SSEA-3, TRA-1-61, TRA-1-80, the transcription factor Oct4, alkaline phosphatase, and telomerase, but not SSEA-1(26, 27). They can propagate indefinitely *in vitro*, have a stable karyotype, and are pluripotent, meaning that they are capable of differentiating into progeny from all three germ layers (28). When engrafted unmodified into immunocompromised mice, both mouse and human ES cells form teratomas and teratocarcinomas comprised of tissue types from endoderm, mesoderm, and ectoderm (2). Since the initial derivation of a human ES cell line in 1998,

Table 1. Selected references of current differentiation protocols

Differentiation Aim	Cell Type Generated	Selected References
Neural	Neurons, oligodendrocytes	32-43
Hepatic	Hepatocytes	44-47
Cardiac	Cardiomyocytes	48-60
Pancreatic	Insulin-producing cell clusters	61-70
Blood	HSC, immunocytes, red blood cells, thrombocytes, myeloid cells	71-77

a large number of additional lines have been generated in countries all over the world, including 61 unique lines available through the National Institutes of Health (NIH) for scientific investigation and 24 lines through the Medical Research Council (MRC) Stem Cell Bank in the UK (29-33). The maintenance of ES cells in culture remains an important first step towards the eventual induction of differentiation towards desired cell types that can be used for therapy. Studies comparing the culture of hES cells in serum-containing versus serum-free media showed that serum was not required to maintain pluripotency (27), and even appeared to be detrimental (34). When human ES cells were cultured in the presence of xenoprotein, they expressed an immunogenic nonhuman Neu5Gc sialic acid (35), although the expression of this sialic acid decreased to less than 1% of cells with the use of B27/N2 supplementation of the medium during induced neural differentiation (36). These studies highlight the possible importance of deriving fresh human ES cell sources that have never been in contact with animal products, and will never be cultured using the traditional methods of animal sera and animal feeder layers. Attempts have also been made to generate human ES cell clones quickly and efficiently via FACS sorting a parent ES cell line (37). Taken together, the data highlight the importance of continual improvements in the derivation and maintenance of ES cell lines so that these initial steps may be optimized.

2.4. Cell replacement therapy and transplantation

The pluripotentiality of ES cells makes them a natural candidate for a broad range of cell replacement therapies, and their unlimited supply offers a solution to the donor shortages currently plaguing all organ transplantation programs. Diseases in which a single cell type is destroyed, or fails to function adequately, are optimal targets for ES cell-based therapeutic strategies. For example, efforts have been made to induce ES cell differentiation into dopaminergic neurons for the treatment of Parkinson's disease; hepatocytes for the replacement of cirrhotic liver; beta cells to reverse type 1 diabetes; cardiomyocytes to regenerate infarcted myocardium; and a host of other cell types with a diverse range of applications. This review will focus on the various transplantation strategies currently under investigation, the successes to date, the problems which have been encountered, and the potential solutions which have been attempted (Table 1). Currently, ES cells are far from realizing their full clinical potential; but with continued progress in both experimental and clinical studies, it seems likely that they will one day play an effective role in the treatment of human disease.

3. TRANSPLANTATION OF EMBRYONIC STEM CELL-DERIVED TISSUE: CURRENT STATE OF THE ART

3.1. Stem cells into neurons

There already exists a large body of data investigating the potential of ES cells in neurodevelopmental biology, with an aim to treat a plethora of neurological deficits resulting from the degeneration of neurons and other neural lineage cell types. Reports have surfaced detailing the derivation of dopaminergic (DA) neurons from human ES cells (38), and their subsequent potential in the treatment of Parkinson's disease (39, 40). Recently, the transcription factors Nurr1 and Pitx3 were found to synergistically promote differentiation of mouse and human ES cells into midbrain DA neurons (41); and ES cell-derived DA neurons showed the ability to partially reverse behavioral deficits in a Parkinsonian rat model (42). ES cell technology has also been applied to the study of Alzheimer's disease, which most often results from the degeneration of cholinergic neurons in the nucleus basalis of Meynert (NBM), with subsequent loss of the basal forebrain cortical cholinergic innervation. Transplantation of ES cell-derived neurospheres into the prefrontal and parietal cortices of NBM lesioned mice dramatically alleviated cholinergic deficits and partially reversed memory loss (43). In a multiple sclerosis study, an interleukin-6/soluble interleukin-6 receptor (IL-6/sIL-6R) fusion protein was used to improve the myelinating capcity of ES cell-derived oligodendrocytes upon transplantation into brain slices of myelin basic protein (MBP)-deficient shiverer mice (44). The regeneration of neurons to treat ischemic brain injury and reverse paralysis has also been studied. One such investigation used neuralized monkey ES cells to engraft into ischemic brain, and showed evidence of neural network reformation (45). In a complementary rodent study, transplanted ES cells were also shown to aid in the regeneration of neuromuscular junctions, providing a partial restoration of motor neuron circuits and a partial recovery from paralysis in adult rats (46). Furthermore, attempts have been made to apply ES cell therapeutics to auditory loss and vision impairment. These sensory deficits, although not life-threatening, still represent debilitating circumstances which have significant negative impact on quality of life. For example, ES cells have been transplanted into the adult vestibulocochlear nerve to correct hearing loss due to sensorineural damage and restore function to spiral ganglion neurons (47, 48); and neuralized ES cells have shown the ability to incorporate into degenerating retina and enhance survival of photoreceptors (49). Taken together, the current picture of ES cells differentiating into neural cell types seems promising. A broad range of clinical conditions are under

investigation, and many experimental successes have already been reported. Although the ultimate move to clinical application is still more a dream than reality, the recent efforts inspire confidence that this goal can one day be achieved.

3.2. Stem cells into liver and heart

In the world of ES cell research, differentiation into hepatocytes and cardiomyocytes has also enjoyed significant scientific scrutiny. The potential applications in degenerative liver disease and infarcted myocardium are obvious. Hepatocyte-like cells have been derived from both mouse and human ES cells (50, 51). Furthermore, normal physiological functionality of these cells was suggested both *in vitro* and *in vivo* via albumin and urea production, and survival and incorporation into mouse liver (52). More recently, a three dimensional scaffolding system was used to generate immature hepatocyte-like cells in culture which, upon transplantation, developed a mature phenotype (53). Hepatogenesis via ES cells has particular significance because although isolated human hepatocytes can be used temporarily as a bridge to transplantation, the liver is one organ which has yet to be replaceable by artificial means. The ability to regenerate liver tissue without resorting to transplanting donor organs would be an enormous step forward in the treatment of cirrhosis. Similarly, studies into the efficacy of ES cell-based techniques for heart repair have also been encouraging. Advances in cardiogenesis continue to be established (54-56), with one study reporting the efficient electrical integration of cardiomyocytes derived from human ES cells into the hearts of recipient pigs (57). Improvement of contractility, and reduction of myocardial wall thinning was shown when ES cells were implanted into ischemically injured myocardium (58). Green flourescent protein (GFP)-labelled ES cells directed towards a cardiogenic fate have shown the ability to both incorporate into myocardium when directly injected into the heart, and home to areas of myocardial infarction when infused intravenously (59-61). Research into the factors that enhance ES cell-derived cardiomyocyte engraftment and function *in vivo* have yielded a panel of growth factors which seem to have beneficial effect when co-injected, including VEGF, FGF, TGF, G-CSF, and insulin-like growth factor (62-64). Recently, in an attempt to determine the most suitable stem cell type for efficient and functional cardiogenesis, Kolossov *et al.* compared bone marrow-derived cells with ES cells and found that only ES cells had the potential to restore contractile function to the infarcted myocardium of syngeneic mice (65). Clearly, investigation into cardiac regenerative medicine is rapidly expanding. With current differentiation strategies being optimized, recent work has focused on the details of delivery and function *in vivo*. Although ES cells have yet to go from bench to bedside, it is encouraging that many clinical trials of "cellular cardiomyoplasty" are already underway, utilizing a range of different stem cells sources (reviewed in (66)). However, as suggested above, ES cells may prove to be a favorable candidate for future clinical application.

3.3. Stem cells into pancreas

In addition to the studies already mentioned, much effort has been directed towards the differentiation of stem cells into pancreatic tissue, in the hope of one day offering cell replacement therapy for type 1 diabetes mellitus. One of the first studies to utilize a systematic culture protocol to generate insulin-producing cell clusters (IPCCs) was published in Science by Lumelsky *et al.* in 2001 (67). This protocol involved a five stage process, with one of the stages relying on the selection of cells expressing nestin, a neurofilament protein. At the end of the final stage, the authors claimed to have generated islet-like tissue that stained for insulin. This report was followed shortly by a study in PNAS from Hori *et al.* (68) where the authors modified the previously published protocol by adding an inhibitor of phosphoinositide-3-kinase (PI3K) during the final stage of culture. This modification enabled an increase in insulin production (68). Concurrent with these publications, other studies also showed the ability to generate insulin secreting cells from both mouse and human ES cells (69, 70). As with all new fields of study, however, controversy soon surfaced. A report published in 2003 claimed that previous *in vitro* generated IPCCs did not actually produce insulin *de novo*, but rather absorbed it from the culture medium (71). C peptide, a by-product of insulin processing, could not be detected, and cells staining for insulin appeared apoptotic or necrotic (72). In the midst of these antagonistic reports, a new culture approach was attempted, which did not rely on a nestin selection stage as had previously been reported. Published in 2004 by a group headed by Anna Wobus, this protocol derived IPCCs and claimed that they had the ability to reverse streptozotocin-induced diabetes in mice (73). Most recently, a five step culture protocol was described by D'Amour *et al.* which enabled the directed differentiation of human ES cells into pancreatic endocrine cells expression insulin, glucagon, somatostatin, pancreatic polypeptide, and ghrelin. Insulin release in response to glucose stimulation, however, was considered minimal (74). As things now stand, it seems likely that *in vitro* derived IPCCs may actually release insulin due to a combination of uptake from the medium and *de novo* synthesis (75).

Due to the controversies outlined above, we made efforts to examine the issue from a different perspective (AB, DCW, KJW unpublished obvervations). Therefore, we first modified and compared three published protocols to find the most efficient method for the generation of IPCCs. Using the mouse ES cell line ESF 122 (CBA-derived, $H2^k$), we subjected the cells to either a 5-step differentiation protocol incorporating a nestin-selection step with (68) or without (67) final development in the presence of a PI3K inhibitor or a 4-step non-nestin selection protocol (76). We then systematically characterised end stage IPCCs by immunohistochemistry, reverse-transcriptase-PCR (RT-PCR) and glucose stimulation/insulin secretion assays at various stages throughout their development.

Immunohistochemical analysis showed higher levels of insulin and c-peptide staining in Wobus-protocol derived IPCCs with approximately 30-40% of cells in each section examined expressing insulin and 10-35% expressing c-peptide. In the Hori protocol 10-30% of cells expressed insulin and 10-20% expressed c-peptide. The

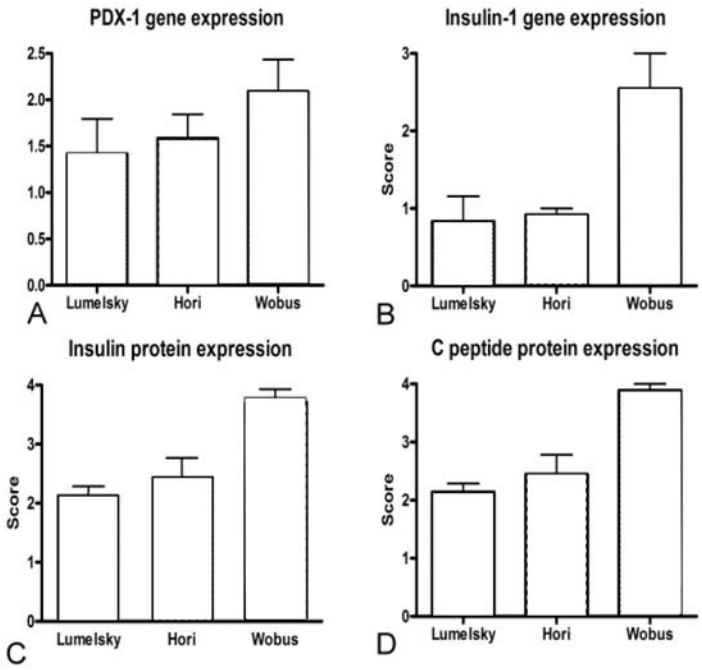

Figure 1. A comparison of three different differentiation protocols. The Wobus protocol derives IPCCs with superior performance in terms of insulin, c-peptide, and PDX-1 expression. Gene expression was measured via semi-quantitative RT-PCR relative to the housekeeping gene HPRT (A, B). Protein expression was measured by freqency and intensity of flourescent staining on immunoflourescence, and scored blindly by two separate investigators over three separate experiments (C, D).

Wobus and Hori protocols produced IPCCs which also expressed some glucagon. In contrast to the Wobus and Hori methods, Lumelsky IPCCs expressed attenuated levels of insulin and c-peptide but significant amounts of glucagon. RT-PCR performed on IPCCs generated using all three protocols revealed, in order of decreasing expression, glucagon, amylase, insulin-2 and insulin-1. Expression of insulin-1 and the pancreatic transcription factor pancreatic duodenal homeobox-1 (Pdx-1) was higher in Wobus and Hori than in Lumelsky-generated clusters (Figure 1). Currently, we are using the quantitative real-time PCR to determine the precise levels of insulin-1, insulin-2 and Pdx-1 in individual IPCCs.

For further studies we selected the Wobus protocol. Transplantation of Wobus IPCCs into the sub-capsular renal space of mice rendered diabetic using the beta cell toxin streptozotocin resulted in a prolonged reversal (> 14 days) of hyperglycaemia in 33% of recipients. In the remainder of recipients, we observed a decline in blood glucose level for at least 5-6 days.

Another method of enriching for insulin producing cells from mouse ES cells was undertaken by Soria *et al.* whereby the neomycin resistance gene was placed under the control of the insulin promoter (69). When exposed to the antibiotic G418, only insulin+ cells survived, and this population proved very efficient in reversing a chemically-induced diabetic hyperglycemia for greater than 11 weeks (69). The diverse range of culture and non-culture techniques used currently in the attempt to generate pancreatic tissue from ES cells highlights the amount of further work yet to be done. The efficient production of glucose-responsive insulin producing cells from ES cells has proven quite difficult, but future study will likely yield more fruitful results.

3.4. Stem cells into blood

The experimental differentiation of ES cells into blood cells of various types is relatively well established (77-80). Currently, human ES cells can be efficiently directed towards a hematopoietic progenitor fate (81), and these cells have recently been shown to have the ability to engraft xenogeneically into recipient sheep (82). Moreover, when ES cells were co-cultured with OP9 bone marrow stromal cells, they differentiated into CD34+ cells with both genotypic and phenotypic similarities to hematopoietic stem cells, and upon further culture could be differentiated into B cells, natural killer cells, macrophages, and granulocytes (79). Human embyronic stem cells have also been used to generate functional T lymphocytes (83). These investigations further the cause of using embryonic stem cell strategies in the replacement or regeneration of all manner of blood cell types. They also highlight the potential of immune tolerance induction strategies that are based on hematopoietic microchimerism.

4. FACTORS HINDERING THE CLINICAL APPLICATION OF EMBRYONIC STEM CELLS

4.1. Embryonic stem cells and tumorigenesis

Although the above studies engender a certain optimism regarding the possibility for ES cell therapeutics, the current reality is that ES cells are still far from the

clinic. In fact, the two characteristics that make ES cells so attractive for cell replacement therapy – their pluripotentiality and their unlimited ability for self-renewal – stand simultaneously as the most daunting obstacles to their successful use in the treatment of human disease, as they translate into haphazard differentiation and tumorigenesis. Teratoma and teratocarcinoma formation *in vivo* probably remains as the single greatest hurdle to successful ES cell-based therapies. Without rigorous elimination of this possibility, clinical transplantation of ES cell-derived progeny will never be safe. Evidence of tumor-forming potential is robust and seen widely across all differentiation protocols, but efforts have been made to address this problem. The diverse strategies that have been utilized include FACS sorting based on immature markers, modification of culture protocols, DNA microarry assays, and genetic manipulation. For example, selecting the SSEA-4 negative fraction of cells via cytometry abolished the incidence of tumor formation when cynomolgus monkey ES cells were used to generate hematopoietic precursors for transplantation into fetal liver (84); and FACS sorting for cells expressing the neural precursor marker Sox1 prior to implantation eliminated tumor forming potential *in vivo* when mouse ES cells were transplanted into mouse brain, allowing for the unfettered development of dopaminergic neurons (85). Another attempt to overcome tumorigenesis focused on a change of culture conditions, which made use of substrate adherent ES cell derived neural aggregates. This procedure led to the disappearance of tumor formation at 4 months post-transplant into degenerated mouse striatum (86). On the other hand, DNA microarray technology was used in an attempt to distinguish undifferentiated human ES cells from their differentiated progeny, with the aim to reduce teratoma formation for future clinical application (87). Finally, in an attempt to control unwanted differentiation and tumor formation, ES cells were knocked out for the EGF-CFC protein Cripto and then induced to differentiate along a neural pathway. The resulting differentiated cells showed the ability to improve the behavioral deficits in a Parkinsonian rat model and did not develop into teratomas (88). Taken together, the established evidence clearly indicates that the tumorigenic potential of ES cells is a serious impediment to their eventual therapeutic use. Nonetheless, recent strategies to combat this problem have met with encouraging success.

4.2. Immunological factors impacting potential embryonic stem cell therapeutics

Another obstacle that remains to be fully elucidated is the potential immune response to an ES cell-derived tissue graft. Skin graft studies in murine models have proven valuable in evaluating the immune response to self and non-self tissue. In a typical transplantation setting, graft rejection is primarily a cell-mediated immunological response. This is demonstrated by the fact that nude mice, which lack T cells, are unable to mount a rejection response against an allogeneic skin graft. Ability to reject the skin graft, however, is restored by adoptive transfer of T cells (for review see (89)). Rejection occurs because of allelic differences between donor and recipient at polymorphic loci which give rise to histocompatibility antigens. The main determinant of graft rejection is the major histocompatibility complex (MHC), also referred to as the human leukocyte antigens (HLA) in humans. Unfortunately, although HLA matching plays an important role, it alone does not allow for graft acceptance in the clinic (90). The most likely explanation for this phenomenon is the presence of minor histocompatibility antigens. Polymorphic self proteins that differ in amino acid sequence between individuals give rise to minor H antigen differences between donor and recipient (91). These differences are also recognized by the host cellular immunity and rejected, albeit more slowly than full MHC mismatches (92). It remains an open question as to what type of immune response, if any, will be generated against a tissue graft derived from an ES cell.

Two schools of thought have arisen in regards to the recipient immunological interface with an ES cell-derived tissue graft. The first expounds the belief that ES cells and their derivatives may enjoy a certain immune privilege, and therefore may not trigger much of an alloimmune response at all; whereas the second argues that an allogeneic graft grown from ES cells will be rejected in the typical fashion.

To begin with, human ES cells have been reported to express unexpectedly low levels of MHC class I molecules, and undetectable levels of MHC class II molecules (93). As a result, their ability to provoke a proliferative response among naïve allogeneic T cells *in vitro* has been questioned, and the belief in their immune privileged status has perpetuated (94). Indeed, one study reported the engraftment and function without immunosuppression of a murine ES cell-derived population of cardiomyocytes into the hearts of infarcted sheep (95). Despite the optimism these studies engender, rejection of ES cell derivatives in a transplantation setting should still be considered a very real possibility. Firstly, although undifferentiated ES cells have been shown to express low levels of MHC, this does not mean that the differentiated progeny will similarly express such low levels. In fact, when ES cells were allowed to differentiate into embryoid bodies (EBs) *in vitro* or into teratomas *in vivo*, MHC expression increased four-fold and ten-fold respectively (93). Also, even if *in vitro* derived ES cell grafts lack passenger dendritic cells (DCs), allowing them to avoid the direct pathway of transplantation rejection, their cellular products presumably will still be taken up by recipient DCs which participate in the indirect pathway responsible for chronic graft rejection (96). Further, minor H antigens may also play a significant role in determining the fate of an allogeneic ES cell graft. A recent study suggested that ES cell-derived cardiomyocytes, when differentiated *in vivo*, have an increased immunogenicity when transplanted into infarcted allogenic mouse myocardium (97).

Having shown IPCCs capable of short and long-term reversal of diabetes, we also conducted investigations on the immunogenicity of ES cells and IPCCs. Congruent with previously published data examining MHC expression on stem cells (98-100), the ES cell lines we utilised expressed very low levels of class I MHC and did not express class II MHC molecules. Furthermore, IPCCs

derived from ESF 150 and ESF 122 mouse ES cell lines also expressed very low levels of class I MHC molecules and also lacked expression of class II MHC molecules. However, both the ES cells and IPCCs exhibited a capacity to express MHC molecules as judged by the up-regulation of both class I and II proteins in response to *in vitro* inflammatory assault with IFN-γ (ASB and KJW, unpublished observations). Thus, IPCCs derived from our ES cell lines may trigger activation of the immune system when transplanted and this may have consequences for the long term viability and function of the transplanted IPCCs.

4.3. Embryonic stem cells and the induction of immunological tolerance

The unique ability of ES cells to give rise to HSC offers an interesting potential whereby immunological tolerance can be induced via hematopoietic chimerism. In certain transplantation settings, allograft acceptance can be mediated by reconstituting the recipient with donor-specific hematopoiesis (101). As detailed previously, ES cells have been readily induced down a hematopoietic cell fate, generating all manner of blood cells. The derivation of HSC from ES cells gives rise to the possibility of mixed chimerism strategies wherein the recipient is reconstituted with ES cell-derived hematopoietic progenitor cells and then engrafted with tissue derived from the same ES cell source. The ES cell-derived HSC could then home to the bone marrow and mature into dendritic cells which could then particpate in thymic selection, eliminating T cells with alloreactivity against the ES cell graft. Such a setting may be favorable towards inducing a central tolerogenic state towards the normally allogeneic ES cell graft. Hematopoietic chimerism tolerance induction strategies have already been experimentally and clinical proven (102, 103). They remain to be tested in an ES cell case, but the theoretical potential is intriguing.

In summary, the immune response to ES cells and their progeny is still very much an open question, and one that requires solid answers if the progression to the clinic is ever to be made (for review, see (104)).

5. PERSPECTIVE: THE FINAL ANALYSIS

The evidence to date suggests that while ES cells are not ready to debut in the clinic, their enormous potential in regenerative medicine and cell replacement therapy cannot be ignored. Theoretically, ES cells have the ability to generate all the tissues of the fetus; and experimentally, this potential has already largely been fulfilled. The differentiation of ES cells into neurons, hepatocytes, cardiomyocytes, insulin-producing cell clusters, and hematopoietic precursors stands as a remarkable achievement of modern science. Further study will facilitate the subsequent synthesis of science and medicine, leading to the clinical application of ES cell technology. Ultimately, such application may offer an extraordinary new tool in the combat against human disease.

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Abbreviations: AS Adult Stem, B27 serum replacement culture medium supplement, CFC cysteine rich domain, DA dopaminergic, EGF epidermal growth factor, ES Embryonic stem, FACS flourescence activated cell sorting, FGF fibroblast growth factor, G-CSF granulocyte colony stimulating factor, GFP green flourescent protein, hES human embryonic stem, HSC hematopoietic stem cell, IFN interferon, IL-6/sIL-6R interleukin 6/soluble interleukin 6 receptor, IPCC insulin producing cell cluster, MBP myelin basic protein, mES mouse embryonic stem, MHC major histocompatibility complex, MRC Medical research council, MSC mesenchymal stem cell, N2 serum replacement culture medium supplement, NBM Nucleus Basalis of Meynert, NIH national institute of health, Nurr transcription factor, Oct transcription factor, OP macrophage colony stimulating factor-deficient stromal cell line, PDX-1 pancreaticoduodenal homeobox-1, PI3K phosphoinosityl 3 kinase, Pitx transcription factor, RT-PCR reverse transcriptase polymerase chain reaction, SSEA stage specific embryonic antigen, TGF transforming growth factor, TRA embryonic stem cell surface marker, VEGF vascular endothelium growth factor

Key Words: Embryonic stem cell, Transplantation, Cell Replacement Therapy, Regenerative Medicine, Review

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