Induced pluripotent stem cell technology and stem cell therapy for diabetes (Review)

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Abstract. Although diabetes can be managed clinically with the use of insulin injections, it remains an incurable and inconvenient disorder. In the long-term, it is associated with a number of clinical complications, such as cardiovascular disease, resulting in a desire for the development of new methodologies to replace defective cells and provide a lasting normality without the need for drug treatment. Stem cells, including induced pluripotent stem cells, offer the possibility of generating cells suitable for transplantation due to their capacity to differentiate into all tissue lineages. However, many issues must be addressed before this type of treatment becomes a reality, including the need for a greater understanding of the underlying biology involved in the onset of diabetes.

Contents

1. Stem cells and diabetes

Current therapy for diabetes mellitus (DM) only provides limited protection against the late complications of diabetes; additionally, although it permits a relatively normal lifestyle, it is by no means a cure. It is crucial that novel therapies are developed that allow effective cell replacement to restore the normal physiological insulin secretion of patients and to eliminate the need for repeated invasive monitoring and exogenous insulin administration by injection.

For some time, DM has been considered a potential candidate disease for medical cellular therapeutic intervention via the replacement of defective β-cells (1). The source of these new β-cells includes the potential use of cells differentiated from human embryonic stem (hES) cell lines. hES cells are isolated from the inner cell mass of developing human blastocysts sourced from excess embryos produced as a result of *in vitro* fertilisation (2). These cells are immortal, capable of unlimited self-renewal, and therefore offer an infinite supply of scalable cells for transplantation. As these cells possess the ability to differentiate into cells from all three germ layers, they have the potential to generate all cell types from all tissues of the body.

However, there are concerns regarding the use of hES cells as a tool for therapeutic transplantation. First, there are ethical concerns inherent in the use and disposal of human embryos (3). This has caused heated debate and regulation over the use of these cells. Second, hES cells themselves are inherently tumourigenic, giving rise to teratoma formation in animal models (4). Any cells for transplantation differentiated from these cells would therefore need to be completely free of any hES cells from which they were derived. Finally, differences in major and minor histo-compatibility complexes (MHCs) present as antigens on any form of graft tissue cells may trigger an immune response from the host, resulting in the rejection of the graft. Although self-renewing hES cells generally express low levels of MHC antigens (5), these levels are gradually up-regulated during their subsequent differentiation into specific tissue cell types, leading to concerns about the possibility of inducing graft vs. host responses from tissue derived from this type of cell. This may necessitate the creation of a bank of qualified and histo-compatibility-typed hES cell lines for transplantation to address these issues (6).

The ability to generate a supply of pluripotent stem cells directly from the somatic cells of affected individuals would provide the basis of autologous transplantation regimes. This

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would avoid the risk of immune rejection or the requirement for long-term immunosuppressive therapies, as somatic cells from the patient's own body would be treated in a manner to create stem cells that resemble embryonic stem cells. These cells could subsequently be differentiated into the required tissue type (possibly after correction of any genetic defects) and used to treat the patient's defective tissue. In addition to avoiding graft vs. host reactions, this type of treatment would also circumvent many of the ethical concerns associated with the use of embryo-derived cell types. Several methods have been considered in order to induce somatic cells to revert to an embryonic state, making them suitable for further differentiation. Strategies including nuclear transfer, cellular fusion and induced reprogramming with defined factors have all been employed.

2. Nuclear transfer techniques for patient-specific stem cells

Nuclear transfer was first described in 1952 by Briggs and King, (7) who demonstrated the creation of normal hatched *Rana Pipiens* tadpoles following the transfer of nuclei from blastocysts into enucleated eggs. Although many examples of successful embryonic nuclear transfer have been reported, it proved difficult to successfully accomplish nuclear transfer from a differentiated mammalian adult cell. A major breakthrough was accomplished by the Wilmut group in 1996, with the creation of 'Dolly the sheep' from nuclei derived from cultured adult mammary gland cells (8). Further mammals have since been successfully cloned, including mice, cows, goats, pigs, rabbits and cats (9). While the production of adult animals from this method remains somewhat inefficient, by contrast the frequency of the derivation of mouse ES cells from blastocysts created by nuclear transfer appears similar to that of ES derivation from natural conceptus (10). This has led to the theoretical possibility of creating 'patient-specific' ES cells through the transfer of a somatic cell nucleus from a patient into a human oocyte. Although breakthroughs in this area have been reported and retracted, nuclear transfer remains an active area of stem cell therapeutic research (16).

The main obstacle to using nuclear transfer to generate 'patient-specific' ES cells is the limitation associated with the access to donated human oocytes. An alternative approach that has been considered is reprogramming via the fusion of somatic cells with previously isolated hES cells. This rationale is an extension of that involved with standard cloning, but in this case via the use of an existing hES cell. The first demonstration of this technique involving human ES cells was in 2005 by Cowan *et al* (11), who demonstrated that the fusion of human fibroblasts with hES cells resulted in hybrid cells with similar morphology, growth rates and antigenic expression to hES cells. The original fibroblasts were marked with antibiotic resistance genes via retroviral transduction in order to allow easy selection of any resultant hybrid cells. The hybrid cells were found to be tetraploid containing both somatic and hES cell chromosomes. The main limitation of the therapeutic use of these cells is thus due to the continued presence of hES chromosomes. This issue has been addressed to a degree by the development of techniques to eliminate specific chromosomes from the resultant hybrid cells. Matsumura *et al* demonstrated such a technique with the removal of specific ES cell-derived chromosomes from fused hybrid cells using genetic targeting techniques (12).

There remains much work to be accomplished on ES cell fusion techniques if this technique is to provide a viable clinical resource. There is a strong possibility of rejection following transplantation of tissues generated from these cells due to persistent expression of ES cell antigens in the resultant hybrid cells (13). Improved techniques capable of removing the entire complement of ES cell chromosomes and avoiding recombination between host and transferred chromosomes are required.

3. iPS cells – cellular reprogramming by defined factors

It has long been hypothesized that ES cells may contain specific and dominant genes or factors that enable them to self renew and maintain their pluripotent state. Indeed, the ES fusion techniques listed above support this hypothesis, as ES cell/somatic cell fusion results in cells that maintain a pluripotent phenotype via the reprogramming of somatic chromosomes (14). Proceeding from this hypothesis, it was considered possible to induce and maintain pluripotency by the introduction or activation of specific factors within somatic cells. The first groundbreaking study that employed this theory was published in 2006 by Takahashi and Yamanaka (15). In this study, the group systematically overexpressed genes in mouse embryonic fibroblasts (MEFs) that were known to be expressed in ES cells in order to assess their ability to induce pluripotency. An assay based on the expression of drug resistance linked to the expression of the Fbx15 gene (an identifier of stem cell pluripotency) was used to assess the effects of each of the factors when introduced to the fibroblasts by retroviral transduction. No single factor was capable of inducing pluripotency. However, when pooled, the overexpression of these genes led to the production of multiple ES-like cell colonies. By a gradual reduction in the number of factors used, four factors were ultimately determined to be the key modulators involved in the reprogramming of these ES-like cells. These genes were Oct3/4, Klf4, Sox2 and cMyc. The resultant ES-like cells were designated 'induced pluripotent stem' (iPS) cells. These iPS cells were analysed by RT-PCR and DNA microarray studies in order to compare marker gene expression, and were found to express the majority of specific ES cell marker genes. Recent evidence does suggest a degree of difference between iPS and ES cells at the transcriptional level (16), and there is some evidence of retention of somatic gene expression from the source cell type (17). The pluripotency of these cells was confirmed by their ability to form teratomas, where the cells differentiated into all three germ layers, with evidence of neural tissue, cartilage and columnar epithelium. However, an inability of the created mouse iPS cells to produce adult chimera mice and subsequent germline transmission led to concerns that the cells were not fully reprogrammed.

In 2007, three groups generated iPS cells that were capable of generating both adult and germline chimeras (18-20). These groups used Oct4 and Nanog activation to select for pluripotency, which resulted in cells that were both epigenetically and biologically indistinguishable from normal ES cells. The Meissner method (18) involved selecting colonies based

upon cell morphology and eGreen fluorescent protein (eGFP) expression instead of using drug resistance as in the Takahashi model (15). Meissner *et al* also demonstrated the creation of an iPS cell line from genetically unmodified mice that was capable of generating chimeric mice. Wernig *et al* used mouse embryonic fibroblasts carrying a neomycin resistance marker present in either the endogenous Oct4 or Nanog (20). This group also demonstrated that, although pluripotency is initially established by virally transduced factors, this pluripotency is mainly maintained by the activity of endogenous pluripotency factors as the viral factors are largely silenced by *de novo* methylation. Okita *et al* (19) demonstrated iPS cell generation using the four retroviral factors following selection for Nanog. As with the other groups, these cell colonies were capable of generating adult chimera mice and were transmitted through the germline. Approximately 20% of the adult chimeras formed from these cells developed tumours, possibly due to the reactivation of the c-myc retroviral transgene. This propensity would obviously limit the potential clinical applications if the above methods were used to generate iPS cells to be differentiated into transplantable cells.

Soon after the above studies, iPS cells were again generated from human fibroblasts by Takahashi and Yamanaka using the same four factors and a retroviral transduction method in order to induce pluripotency in adult human dermal fibroblasts (21). In this case, to increase the transduction efficiency, the mouse retroviral receptor Slc7a1 was introduced into the fibroblasts via a lentiviral vector prior to retroviral induction with the four factors. This resulted in the formation of ES-like cell colonies at day 30. The human iPS cells created in this way were morphologically similar to hES cells and had similar surface markers, gene expression, telomerase activity, *in vitro* differentiation and teratoma formation. There was evidence that the retrovirus expression was strongly silenced following transduction, however, there also was evidence of at least 20 retroviral integration sites per clone (approximately three to six for each factor introduced). Park *et al* (22) induced iPS cells from human ES cell-derived fetal fibroblasts (in this case differentiated ES cells expressing GFP and neomycin resistance genes integrated into the OCT4 locus). The cells were infected with a cocktail of retroviral supernatants from the four factors. Following this, they generated iPS cells from primary fetal tissue as well as adult fibroblasts. In these studies, OCT4 and SOX2 appeared to be essential for reprogramming with either KLF4 or cMyc enhancing the efficiency of colony formation. Yu *et al* (23) also successfully demonstrated the reprogramming of human somatic cells to pluripotency, but accomplished this by using OCT4, SOX2, Nanog and Lin28.

Therefore, Oct4 and Sox2 appear to play a pivotal role in the generation of iPS cells, with other factors being interchangeable. Huangfu *et al* (24) successfully generated iPS cells from primary human fibroblasts using retroviruses expressing only Oct4 and Sox2 in an optimized protocol exposing the cells to valproic acid (a histone deacetylase inhibitor shown to improve reprogramming efficiency in MEFs). This has potential clinical application advantages due to the elimination of two potent oncogenes from the transduction process. In 2009, Kim *et al* (25) demonstrated single factor induction of iPS cells by using only Oct4. In this case, adult mouse neural stem cells were used as the source cell type. Currently, single factor induction has not yet been reported in human cells, although this certainly will be a major area of research.

Other reprogramming methods have been successfully implemented following the original retroviral methods. Virus-based reprogramming methods result in varying degrees of viral integration into the somatic genome. This obviously carries with it safety concerns over endogenous gene activation or inactivation as well as transgene persistence or reactivation after differentiation. A single iPS clone has 20-40 viral integration sites, and although it may be possible to perform whole-genome sequencing to map these sites, it would prove difficult to ensure their clinical safety (26).

Since the initial retroviral-mediated generation of iPS cells, several groups have successfully employed other methods of reprogramming somatic cells to the pluripotent state, including plasmids (27), lentiviruses (28), excisable transgenes (29,30), recombinant proteins (31) and episomes (32).

iPS cells created by plasmid transfection have been reported by Okita *et al* (27). In this report, the group used two individual plasmid vectors containing the CAG promoter, with one containing Oct4, Klf4 and Sox2 and the other containing cMyc. Using a transfection protocol targeting embryonic fibroblasts on days 1, 3, 5 and 7, they successfully created mouse iPS cells, with no evidence of the genomic integration of plasmid DNA. These clones successfully generated teratomas and chimeric mice. Soldner *et al* (28) made use of doxycycline-inducible lentiviral vectors of the four reprogramming factors, which were excisable with Cre-recombinase. By this method, a total of 16 clones were generated, with no evidence of integration of any of the viral reprogramming factors.

Recently, two groups have used excisable transgene technology to allow virus-free integration of the programming factors, followed by their subsequent removal (29,30). Both groups made use of the 2A peptide sequence from foot and mouth disease virus to link the sequences of cMyc, Klf4, Oct4 and Sox2, allowing efficient multiprotein expression from a single vector. Transduction to the pluripotent stem cell state was first achieved by introduction of the single vector 2A linked system. The exogenous factors were then removed by transient Cre transfection, as the reprogramming cassette was flanked by loxP sites. A piggyBac (PB) transposon was then used to deliver the reprogramming factors under the control of a doxycycline-inducible system. The PB system allows for the removal of the transgene upon re-expression of the PB transposase, thus creating iPS cells with no trace of reprogramming factors once the exogenous expression is no longer required.

Zhou *et al* (31) recently described the generation of mouse iPS cells using recombinant proteins. This technique avoids the use of any genetic material, potentially reducing the likelihood of unexpected genetic modifications to the somatic genome. In this case, the reprogramming proteins are delivered directly into the cell, as opposed to relying on the cell machinery to transcribe the proteins itself. This study used *E. coli* bacteria to generate inclusion bodies containing the proteins, which were subsequently purified. The resultant proteins were then used to reprogram MEFs, resulting in induced pluripotent stem cells. There are potential advantages to this technique, as there is no need to analyse and select the resultant colonies based upon the existence of integration sites in the somatic genome.

It has also recently been reported that certain small molecules can be used to both enhance the reprogramming process and, in some cases, to replace specific factors. Shi *et al* (33,34) identified small molecule combinations that significantly enhance the reprogramming process (using only Oct4/Klf4) in MEFs. Other groups have also shown enhanced efficiency with the additional use of small molecules that are known to inhibit specific pathways within the cell (24,35). There is also a desire to carry out iPS generation using small molecule techniques alone.

The final method employed for the generation of human iPS cells completely devoid of vector and transgene sequences has been the use of episomal vectors based on the EBNA1/oriP vector, which is derived from the Epstein-Barr virus (32). These vectors are gradually lost from proliferating cells, resulting in a fraction of subclones showing a complete absence of both vector and transgene sequences. Although this method currently results in relatively low reprogramming frequencies, this will likely be improved by the addition of other chemical compounds and small molecules shown to increase efficiency with the other modalities.

4. Generation of β-cells from iPS cells

ES cells have the ability to generate cells of any tissue type. This makes them candidates for creating functional β-cells, which could potentially be used as a transplantable tissue source for diabetic patients. Initial attempts at this type of differentiation using mouse ES cells used a transfection and selection method to screen for insulin-secreting ES cell clones capable of restoring normoglycaemia in diabetic mouse models (36). Lumelsky *et al* (37) selected cells positive for nestin (a filament protein found in neural precursors) following embryoid body formation. Following further differentiation, cells that resembled islet cells were produced. However, repeated analysis of such protocols by other groups suggested that these cells were not capable of *de novo* insulin synthesis to the same degree as normal β-cells, and in fact were principally secreting insulin absorbed from the culture media.

It became clear that a more stepwise approach to β-cell generation was required, capable of reflecting the changes observed in normal human development. In these protocols, the initial differentiation step is designed to drive differentiation towards the formation of definitive endoderm. Developmental studies have revealed that the formation of endoderm during normal fetal development is instigated by Nodal (a member of the transforming growth factor-β). Using Activin A, which is closely related to Nodal, two groups successfully generated endodermal cells (38,39). Following this, one of these groups continued with a stepwise differentiation protocol, based on normal development through definitive endoderm, primitive gut tube, posterior foregut, pancreatic precursor and endocrine cells (40). The cells generated by this protocol contained similar amounts of glucose to normal adult β-cells. When exposed to secretory stimuli, these cells were also capable of releasing insulin. However, the lack of response of these cells to glucose suggested that they may be akin to immature fetal β-cells rather than mature β-cells found in the adult. In 2007, Jiang *et al* continued to develop a step-wise approach from definitive endoderm in serum-free conditions (41). This protocol resulted in the production of insulin-producing islet-like clusters containing cells representative of ductal, exocrine and endocrine pancreas. These β-like cells contained secretory granules and responded to an *in vitro* glucose-stimulated release assay. In 2008, Kroon *et al* successfully generated human endoderm capable of producing insulin that was responsive to glucose in such a way as to achieve normoglycaemia in a diabetic mouse model (1). In this case, the endoderm was implanted into mice prior to terminal β-cell differentiation. These mice were then rendered diabetic by the destruction of their natural β-cells. Animals with the grafted endoderm maintained normal blood glucose levels, while control animals became hyperglycaemic. When the grafts were removed, the grafted animals were also rendered hyperglycaemic. Although this research is extremely promising, over 15% of the animals developed tumours in the graft, raising safety concerns for translation in humans.

Currently, there are two successful reports of insulin-producing cells generated from iPS cells (42,43). Tateishi *et al* (42) used retrovirally induced iPS cells sourced from human skin cells. These cells were then subjected to a protocol almost identical to that of Jiang *et al* (41) above. This resulted in the formation of glucose-sensitive insulin-secreting cells. Following this, Zhang *et al* (43) published a protocol similar to Jiang *et al* to differentiate ES and iPS cells in a step-wise fashion, resulting in approximately 25% of cells positive for insulin. These cells secreted C-peptide in response to glucose stimulation *in vitro*. Maehr *et al* (44) also demonstrated the generation of iPS cells from the skin biopsies of patients with Type 1 DM, with subsequent differentiation of these cells into insulin-producing/glucose-responsive cells.

5. Future directions

Significant steps have been made towards the use of stem cell technologies to generate β-cells as a potential therapy for patients suffering from diabetes. However, many hurdles must be overcome to generate a safe clinical application from this technology. iPS cell technology may provide the potential for ethically acceptable replacement tissue that is free from the problem of immunological-mediated rejection. Studies demonstrate that iPS cells clearly have the potential to differentiate into β-cells, although better and more efficient techniques, as well as more robust and more extensive characterisation of the differentiated cells, must be addressed. Despite these advances, the possibility of tumour formation caused from contaminating residual stem cells after differentiation that may lead to teratoma formation remains a concern. Moreover, the inherent risk of aberrations caused by reprogramming must be carefully studied. If retroviral integration is used, then there are concerns about the effects of these integrations on the host genome, as well as the use of oncogenic viruses. To this end, even greater importance will be placed on studies to develop safer methodologies of reprogramming, which are already well underway. This rapidly developing iPS technology offers a promising route for finding a cure for this widespread, costly, and eventually destructive disease. Clearly, this approach must be coupled with endeavours to obtain a comprehensive understanding of the factors responsible for the onset of diabetes in any particular patient. Even when

β-cells for transplantation can be efficiently constructed from stem cells, a greater understanding is required concerning both autoimmune reactions that lead to β-cell destruction and those inherent β-cell defects that lead to reduced functionality in order for this technology to be applied.

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