Challenges and Prospects for Stem Cell-Based Therapy in Diabetes Mellitus

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Diabetes mellitus is a serious health problem worldwide. DM in all its forms currently affects at least 200 million people in the world, and this number is expected to rise to more than 350 million by the year 2030 [1]. About 10% of diabetic patients suffer from type 1 diabetes. They are treated mainly with insulin. Beta cell replacement therapy could, in principle, provide a cure for these patients as well as for rare cases of maturity-onset diabetes of the young. Thus, pancreas and islet transplantation have been impressive in rendering some patients insulin-independent for a number of years [2]. It is now well accepted that β cell mass is decreased by about 50% in type 2 DM [3]. Replacing these missing cells could be a means to treat the insulin-delivery malfunction characteristic of this form of DM as well. But this requires possibly toxic immunosuppression and there will never be enough donor islets to satisfy demand.

CELL REPLACEMENT THERAPY

Several potential approaches for cell replacement therapy have been investigated, including various modified rodent and human β cell [4] and non-β cell lines [5,6]. More recently, attention has focused on stem cell research since stem cells, both of adult and embryonic stem cell origin, hold much promise of providing a potentially unlimited source of replacement cells. Functionally defined, stem cells have the dual capacity both to self-renew and exhibit multilineage differentiation.

Recent reports have led to the emerging concept that stem cells may exhibit extraordinary plasticity in their differentiation repertoire, including the production of cell types outside of the organ system in which they reside [7,8]. It is increasingly recognized that the apparent heterologous cell fates might be explained by the contribution of a donor cell marker to an existing host cell, thus forming a hybrid cell of target tissue phenotype without *bona fide* differentiation of the donor cell [9]. Therefore, the field of generating new β cells from stem cells is still very much a work in progress, and each new report has been met with concomitant excitement and skepticism. Various sources of adult stem cells were reported to have the potential for differentiation into islet endocrine cells, including β cells [10]. They originated either from mesoderm, ectoderm or endoderm and have been cultivated from pancreatic or extrapancreatic tissues and from fetal or adult tissues. Yet, adult stem cells are difficult to harvest in large numbers; they have a relatively more restricted repertoire of differentiation outcomes and a more limited proliferative capacity. These limitations may present significant *a priori* barriers to achieving large numbers of derivative cell types required for human replacement therapy.

DM = diabetes mellitus

EMBRYONIC STEM CELLS

Embryonic stem cells are considered the prototype stem cell. They have proven to be more versatile then adult stem cells. They are derived from the inner cell mass of a preimplantation embryo and were first cultivated from mouse blastocysts in 1981 [11]. In humans, comparable success was achieved a decade ago following the establishment of proper growth conditions [12,13]. The blastocysts were obtained from surplus clinical *in vitro* fertilization products that would otherwise have been discarded and were donated for research by couples following informed consent.

In the undifferentiated state, human ESCs are characterized by a normal diploid karyotype, and cell surface markers of uncertain significance characteristic of human embryonal carcinoma cells and undifferentiated non-human primate ESCs (alkaline phosphatase, stage-specific embryonic antigen-3, SSEA-4, tumor-related antigen-1-60, TRA-1-81) [14]. Moreover, hESCs retain activity of the ribonucleoprotein telomerase, and as a result they never reach senescence following extended periods of propagation *in vitro*. Pluripotency of hESCs stands for their ability to contribute to various tissues derived from the three embryonic germ layers following induction of differentiation, either *in vivo* during generation of teratomas when injected into SCID mice [12], or *in vitro* following removal of the support feeder layer or following aggregation in suspension cultures [15].

ESCs = embryonic stem cells
SSEA = stage-specific embryonic antigen
TRA = tumor-related antigen
hESCs = human ESCs

LESSONS FROM STUDIES ON HUMAN AND MOUSE ESCS

Recent work using murine and hESCs indicated that they may indeed serve as a rational platform to conduct studies regarding lineage commitment, differentiation and genetic modification. As we pursue the novel goal of generation of insulin-producing cells differentiating out of hESCs, it should be borne in mind that the healthy β cell maintains exquisite control of plasma glucose through the release of insulin in the basal state and following stimulation. This finely tuned process must adapt to the numerous changes that occur during an individual lifetime, including aging, infections, pregnancy and more. Thus, in developing human embryonic stem β cell replacement it is imperative that attention be appropriately focused on producing a cell that is capable of responding to a variety of physiological needs in a manner similar to that of authentic β cells on a daily basis and during its lifetime.

Using *in vitro* spontaneous differentiation systems, reverse transcriptase-polymerase chain reaction studies detected transcripts of insulin, key transcription factors in pancreatic differentiation, as well as GLUT2 and glucokinase that are involved in glucose-dependent insulin secretion upon induction of differentiation but not in undifferentiated cells [16,17].

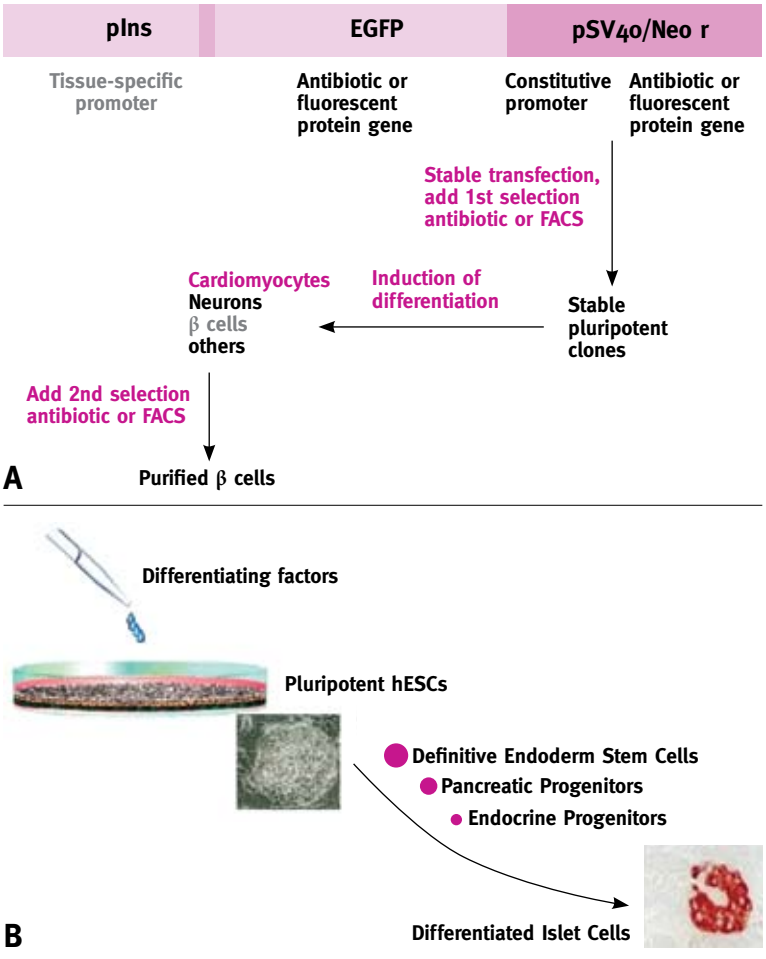
Immunohistochemistry studies, carried out on teratomas, were able to demonstrate insulin-positive cells, but these were in relatively small numbers. Using double immunofluorescence staining, these cells were shown to co-express insulin and C-peptide. Furthermore, double immunostaining with anti-Pdx1 indicated the presence of a larger number of Pdx1-positive cells, lining a gut-like structure, among which the insulin-producing cells were identified (personal unpublished data). Thus, the above findings supported the concept of the inherent capability of hESCs to differentiate into pancreatic β cells. They were a necessary prerequisite for subsequent strategies seeking islet β cell enrichment and further characterization, but rather insufficient to define those insulin-containing cells as presumptive pancreatic β cells. Therefore, major hurdles and challenges are to be overcome until clinical trials can be conducted [Table 1].

Accordingly, enrichment and isolation of endocrine pancreas cells could potentially be achieved, by genetic modification [18] or by exposing the undifferentiated cells to different extracellular matrices and/or successive media containing different growth factors, based on prior knowledge of endocrine pancreatic differentiation [19] [Figure 1]. This may turn out to be prohibitively cumbersome as hundreds of growth and survival factors may be involved. However, several researchers attempted to use such a strategy and showed islet-like structures derived from ESCs [20-22]. Of interest, the enrichment protocol used in these studies follows a neu-

Table 1. Challenges and hurdles in human embryonic stem cell research

1	Enrichment of purified homogenous progenitor or terminally differentiated cells
2	Comprehensive molecular and functional characterization of ES-derived cells
3	Scaling up the number of purified cells to accommodate needs of transplantation
4	Circumvent growth arrest and senescence *in vitro* and *in vivo*
5	Circumvent immune attack
6	Circumvent tumorigenicity

Figure 1. Current experimental strategies used for enrichment, tracking and selection of endocrine islet cells differentiating from pluripotent human embryonic stem cells. **[A]** Schematic diagram of "gene trapping" strategy for cell enrichment. Undifferentiated ES cells are transfected (or infected) with a plasmid vector (or lentiviral) as indicated below. Stable pluripotent clones are resistant to and propagated in the presence of the first-selection antibiotic. Following induction of differentiation, a mixed population of ES-derived cells is obtained. Selection of the desired cell types is achieved by the addition of the second-selection antibiotic. A cell-specific promoter, such as the insulin promoter (pIns), drives the gene conferring resistance to the second-selection antibiotic [18]. Alternatively, cells can be sorted using fluorescence - activated sorting, based on enhanced green fluorescent protein (EGFP, or other fluorescent proteins). **[B]** Schematic of directed differentiation of hESCs via endodermal developmental pathway [27,28]. Undifferentiated hESCs are exposed to various growth conditions at each step, leading to lineage commitment and enrichment of the indicated cells.



roectodermal rather than a conventional endodermal developmental paradigm for differentiation of insulin-producing cells. Reassessment of the previous protocols yielded controversial interpretations. Based on several lines of evidence, we and others concluded that most of the insulin staining was not of endogenous origin but was probably endocytosed insulin trapped from culture media [23], and considered it unlikely that neuroectodermal differentiation would lead to the generation of β cells. Others claimed that two populations of insulin-positive cells exist: one consisting of neurons or neuronal precursors that produced pro-insulin and secreted it in a manner that is not fully physiologically regulated, while the other cells are apoptotic with exogenous or endocytosed insulin trapped within [24,25].

Therefore, efforts have shifted toward recapitulation of the normal embryonal development, with emphasis on endoderm lineage enrichment that can be further coaxed to differentiate into pancreatic lineages. The studies by Baetge and colleagues [26-28] successfully extrapolated this concept to hESCs. They showed differentiation of hESCs into pancreatic cells, of which 7% had authentic β cell characteristics. Their protocol was highly reproducible in our hands. We confirmed the presence of definitive endoderm progenitor cells that expressed telomerase activity, suggesting the possibility of scaling up their numbers probably without reaching senescence (personal unpublished data).

Other recent publications demonstrating that adult β cells are formed by self-duplication [29], refuting the existence of a population of adult stem cells for β cell differentiation and the notion that pancreatic organ size is determined by embryonic progenitor cell pool in mice [30], emphasize that stem cells of embryonic origin (or the more recently described cells of somatic origin into which a set of pluripotency genes have been introduced) [31,32] may be the only source for potential provision of an unlimited source of cell replacement for transplantation.

However, many issues still require thorough investigation:

- Embryonic stem cells: such as safety and tumorogenicity of the transplanted ES-derived cells
- Immunogenicity: both alloimmunity and autoimmunity in the case of cell replacement in autoimmune diseases
- Normal development: comprehensive definition of pancreatic and endocrine stem cell and progenitor characteristics
- Diabetes alleviation: transplantation of islets vs. pure β cells or progenitors, ideal site for transplantation, and many other questions that are widely in debate.

Of note, different approaches involving "gene therapy" instead of "cell therapy" are also being pursued, and may provide an alternative promising approach [33-38].

As clinicians, our principal guideline is "primum non nocere." Novel potential therapies as discussed earlier should pass very high standards prior to application in our patients [39], especially in metabolic and endocrine disorders, since they have been treated effectively by hormone replacement for decades.

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