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FOR DEBATE

Embryonic stem cell-based diabetes therapy—a long road to travel

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Abbreviations

CRT	cell-replacement therapy
EB	embryoid body
ES	embryonic stem

Embryonic stem (ES) cells have been widely heralded as a source of differentiated cells to be used in cell-replacement therapy (CRT), which has potential as a treatment for a number of degenerative diseases, including Parkinson's disease, Alzheimer's disease and diabetes [1–3]. The potential impact of CRT on human life is huge. Not only will healthcare systems be transformed, but a number of societal issues will emerge if cell-based therapy is fully developed. There are, however, widely diverging views as to when we can expect to see cell-based treatment for major diseases (see e.g. [4] in this issue), and over-optimistic views have been expressed in the press and elsewhere. Here I present a more pessimistic view on the amount of time that will be needed to develop CRT for the treatment of diabetes.

Patients with type 1 diabetes are considered to be candidates for cell-replacement therapy (CRT), but those with advanced stages of type 2 diabetes might also benefit. Many obstacles, however, must be overcome before transplantation becomes the treatment of choice for patients newly diagnosed with diabetes. At present, only patients with poor metabolic control and a history of hypoglycaemic episodes are eligible for islet transplantation [5]. This selection is based on an unfavourable risk–benefit analysis in patients who are responding well to their current therapy of multiple daily insulin injections (i.e. patients with metabolic control sufficient to confer a low risk of secondary complications). Indeed, concerns over whether the immunosuppression needed to prevent graft rejection will be associated with serious side effects also limits the widespread use of CRT for diabetes [6]. Unless new forms of immunosuppression or encapsulation technologies with minimal side effects are developed, CRT faces a bleak future in the treatment of diabetes. The risks associated with the immune suppression must be lower than the risks of secondary complications associated with the disease itself before it will be ethically responsible to treat diabetes with CRT. It should not be forgotten that new generations of insulin analogues, and the development of closed-loop insulin delivery systems, may provide the means to improve the clinical outcome of conventional insulin therapy [7], raising further concerns as to whether the safety of future strategies to prevent graft rejection will allow a favourable risk–benefit analysis.

Assuming that safe and efficient methods to prevent graft rejection are developed, a second and equally important obstacle emerges, namely the shortage of organ donors. The number of available donor pancreata per year is currently sufficient to treat only about 1 in 20 of the newly diagnosed type 1 diabetes patients in the USA each year [8] and it is obvious that an increase in donor pancreata sufficient to meet the demand is neither likely nor desirable. An alternative source of islet beta cells is therefore required before islet transplantation can be more

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widely used. Thus, ES cells enter the stage. There are a number of alternatives to ES cells, including expansion of beta cells before transplantation, adult stem cells and surrogate beta cells, but it is beyond the scope of this paper to deal with these. I will focus on the progress and set-backs in the efforts to generate beta cells suitable for transplantation from ES cells.

The first report of insulin-producing cells derived from mouse ES cells was published in 2000 by Soria and co-workers [9]. Using embryoid body (EB) formation in the presence of serum to induce the spontaneous differentiation of ES cells (made possible by the presence of an insulin promoter driving the expression of the neomycin resistance gene) combined with drug selection, a homogenous population of insulin-producing cells was obtained. These cells were able to correct streptozotocin-induced hyperglycaemia in mice when transplanted under the kidney capsule. It is unclear to what degree these cells were proliferating as opposed to being mitotically quiescent, as normal beta cells are. Since a clone was derived, we must assume that these cells were indeed proliferating. Although the transplanted cells did not form tumours within the 6 weeks of the experiment, it remains to be seen whether such cells remain non-tumourigenic in long-term experiments. A recent report by the same group investigated the proliferation of cells obtained in much the same way, and found a BrdU labelling index close to 50% [10], much greater than that of normal beta cells, which typically have BrdU labelling indices of about 0.2%. This high labelling index raises doubts about the normality of these cells. Indeed, 40% of the animals that received cell implants had only a transient correction of hyperglycaemia [9], further suggesting that these ES-derived insulin-producing cells behave differently from normal beta cells. In another study using EB formation to initiate differentiation, Odorico and co-workers developed insulin-producing cells from mouse ES cells, together with several other pancreatic endocrine cell types [11]. The efficiency was, however, very low and the cells were not tested for their ability to correct experimental hyperglycaemia. Studies on human ES cells have had similar findings [12].

In 2001 McKay and co-workers reported that insulin-producing cells could be derived from a population of nestin-producing cells [13]. Minor variations to this protocol have been made, including introduction of the transcription factor gene *Pax4* into the ES cells before differentiation [14] and addition of a phosphoinositide-3 kinase inhibitor to the final step of differentiation [15]. The rationale underlying several of these studies is the observation that development of pancreatic endocrine cells and central nervous system neurons share genetic and developmental pathways [16, 17]. Pancreatic endocrine cells share a number of characteristics with neurons [17], and insulin-producing cells have been observed in the nervous system of several invertebrates [18–20] and in primary cultures of mammalian fetal brain cells [21]. Thus, many protocols for differentiation of ES cells were designed to produce or select for neural progenitors defined by expression of the intermediate filament protein nestin [22] and then to direct pancreatic islet differentiation in subsequent steps.

Subsequent reports have questioned the interpretation of results obtained with protocols based on selection of nestin-positive precursors [23–25]. This difference of opinion was based on experiments designed to test whether the insulin immunoreactive cells observed in the initial studies cells were indeed insulin-*producing* or merely insulin-*containing*. The first observation that hinted at problems with the notion that these cells actively synthesised insulin was that the insulin-positive cells displayed condensed nuclear DNA, and were positive in TUNEL assays [23], suggesting that they were undergoing apoptosis. This idea has subsequently been supported by another independent report [24] and by the detection of activated caspase-3 in the insulin-immunoreactive cells [25]. Second, when insulin present in the culture medium was replaced with FITC-labelled insulin, it became clear that the insulin-immunoreactivity could be explained by the uptake of exogenous insulin by the apoptotic cells [23]. Further, when the insulin-immunoreactive cells were assayed for the release of insulin as well as C-peptide, it was evident that the cells which released readily detectable amounts of insulin failed to demonstrate detectable release of C-peptide [25]. Since C-peptide is synthesised as part of the pro-insulin molecule, it should be released together with insulin in equimolar amounts. Together, these observations suggest that insulin release and insulin immunoreactivity can be explained by uptake of exogenous insulin present in the culture media. Thus, it has been recommended that assays for C-peptide secretion should complement insulin release data in future studies, preferably in combination with other assays for de novo synthesis of insulin, to support conclusions that beta-like cells have been produced by in vitro differentiation protocols [25].

In order to be confident that normal beta cells are generated, one must take into account the embryological origin of the pancreatic islets and recapitulate the normal sequence of molecular signals that occur during embryonic. Since the pancreas, including the endocrine component, is derived from definitive endoderm, current efforts focus on inducing the in vitro generation of cells representing early definitive endoderm. This approach has been taken in a number of recent studies [26–29]. Formation of definitive endoderm occurs during gastrulation, and observations from a number of vertebrate species have demonstrated a crucial role for Nodal signaling in this process, although it is unclear to what degree results from fish and frogs can be

directly extrapolated to mammals [30]. Keller and co-workers used activin, which together with Nodal belongs to the TGFβ superfamily and activates Smad2-mediated intracellular signaling similar to that triggered by Nodal, to induce expression of the T-box gene *T* (brachyury; monitored by a *T*-GFP reporter). *T*-expressing cells can give rise to both definitive endoderm and mesoderm. Critical for the further development of definitive endoderm from these *T*-expressing cells was that the activin treatment was performed in the absence of serum. In the presence of serum, *T*-expressing cells still formed, but yielded only mesoderm upon further differentiation [26].

More recently, a report by Nishikawa and co-workers generated similar bi-potential cells using a goosecoid (*Gsc*)-GFP reporter [27]. This group has now refined their approach and defined a number of cell-surface markers that can be used to separate mesodermal and endodermal cell types [28]. While these studies have been performed on mouse ES cells, Baetge and co-workers have recently reported very similar results with human ES cells [29]. Based on the expression of marker genes, these reports seem to demonstrate convincingly that formation of definitive endoderm can now be reliably achieved for both mouse and human ES cells. Together, these recent reports demonstrate that significant advances have been made in directing ES cells towards definitive endoderm and that we now have better molecular markers that allow selection of the desired cell types. However, none of these studies has included any sort of functional assay to substantiate that endoderm with the capacity to generate specialised endodermal cell types is being formed. Moreover, the cells seem to be quite refractory towards further development into pancreatic cell types in vitro [27].

Thus, it seems that the next challenge will be to differentiate such cells into specialised cell types that can be used for CRT. In order to accomplish this task, the development of novel functional assays may also be required, as well as identification of the molecular signals that govern the specification of pancreatic endoderm and its associated cell types. Much has been learned in recent years [16, 17], but many of the signals are still unknown. A recent study by Semb and co-workers hints that partly differentiated human ES cell progeny are capable of responding to developmentally relevant signals arising from the embryonic pancreas [31]. It is noteworthy that the remarkable progress seen over the past 20 years in the field of haematopoetic stem cells has relied on excellent functional assays (i.e. clonogenic assays), as well as on the availability of a large number of cell surface markers, allowing separation of distinct cells types by fluorescence activated cell sorting (FACS).

In conclusion, before CRT with ES cell-derived beta cells can become a reality, progress has to take place on several fronts: we need to uncover the molecular signals that orchestrate normal embryological development of the pancreas and its beta cells; we must develop novel functional assays to assist in the development of differentiation protocols; we must rigorously test the functionality of the resulting in vitro generated beta cells; we have to invent new and safe strategies for preventing graft rejection. Once these challenging tasks have been solved, the proposed CRT will have to undergo rigorous safety assessment, and obtain regulatory approval, before it can be offered to patients using safe and largely adequate insulin injection therapy. It may take a lot of time to meet all these challenges. However, nothing we have learned so far seems to indicate that this it is not possible. It is therefore only a matter of time before CRT will transform the way we think about treatment.

- In order to make progress on CRT with ES cell-derived beta cells, we need to:
  - uncover the molecular signals that orchestrate normal embryological development of the pancreas and its beta cells;
  - develop novel functional assays to assist in the development of differentiation protocols;
  - rigorously test the functionality of the resulting in vitro generated beta cells;
  - invent new and safe strategies for preventing graft rejection.
- Once these challenges have been addressed, the proposed CRT will need to:
  - undergo rigorous safety assessment;
  - obtain regulatory approval.

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