A journey from the insulin gene to reprogramming pancreatic tissue

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Abstract

This article was written as a contribution to mark the centenary of the first administration of insulin to a human in 1922. Writing from an Aberdeen perspective, an introductory passage will place emphasis on the role of JJR MacLeod, under whose supervision the discovery of insulin by Banting and Best was made. The major thrust of the article, however, will be on the cloning and sequencing of the human insulin gene, and the impact it had on the scientific career of the author. It initiated a journey to find alternative therapies for diabetes that led sequentially though gene therapy, embryonic stem cell-derived islets, and reprogramming. Our experience in these areas will be described, with emphasis on the strengths and weaknesses of each of these approaches.

Br J Diabetes 2022;**22**(Supp1):S72-S78

Key words: gene therapy and diabetes, embryonic stem cells and diabetes, reprogramming and diabetes, pancreatic transcription factors; Islets of Langerhans

Introduction

The discovery of insulin in 1921 was a remarkable scientific achievement, not least because of the breathtaking speed at which it occurred.[1] The experiments, carried out by Banting and Best in the laboratory of JRR MacLeod in Toronto, commenced in May of that year and involved dog pancreatectomy followed by injection of extracts of atrophied pancreas. By the end of the year, the team, now joined by Collip, had an alcohol extract of pancreas which when injected into pancreatectomised dogs led to a reduction in blood glucose levels. The partially purified factor, insulin, was administered to the first human (Leonard Thompson) in January 2022. In 1923 the Nobel Prize in Physiology was awarded to MacLeod and Banting for the discovery of insulin.

In the short period that they worked together on the discovery the relationship between Banting and MacLeod deteriorated markedly. MacLeod had been on holiday while the project was getting underway, and Banting felt that on his return Macleod took possession of the project and took undue credit for the discovery. This led to a great deal of acrimony: Banting thought that Best deserved some of the glory and shared his Nobel Prize money with him, and in turn MacLeod shared his with Collip. The situation reached such a heated level that Banting, Best and Collip were invited by the Board of Governors of the University of Toronto to write their accounts of the discovery. These documents, along with an account supplied by MacLeod, established that the original idea initiating the fundamental research was Banting's but that the work could not have been taken to fruition without the advice, facilities and support provided by MacLeod.[2] A narrative then developed in which MacLeod's role was marginalised – the forgotten man.

As a result of the ensuing unpleasantness, in 1928 MacLeod left Toronto, returning home to Scotland to take up the Chair of Physiology at the University of Aberdeen, his alma mater. His research there was hindered by ill health, and he died at the early age of 59 in 1935. In his will he donated his Nobel Gold Medal and Citation to the University of Aberdeen, where copies are on display in the Institute of Medical Sciences. His will also contributed to funding a Professorial Chair in Biochemistry. The author of this article is proud to be the third holder of the MacLeod-Smith Chair of Biochemistry. Access to archival material has gone a long way to ensuring that MacLeod's contribution to the discovery of insulin is now fully recognised and appreciated.[3]

The centenary celebrations prompt us to highlight some of the major scientific advances since then that have progressed our understanding and treatment of diabetes. These might include the sequencing of the insulin protein by Sanger, the elucidation of the crystal structure of insulin by Hodgson, the development of a radioimmunoassay for insulin by Yalow, and the discovery of proinsulin by Steiner. However, the breakthrough with possibly the most important impact came about through the efforts of three competing groups based on the east and west coasts of the USA. These groups came together to co-publish a landmark paper that described the cloning and sequencing of the human insulin gene.[4] The importance of this scientific breakthrough was that it kick-started a whole new era in diabetes research. To some extent it initiated studies on the genetics of type 1 diabetes (T1DM), and to this day the most important genetic component to the disease can be mapped to the insulin locus on the short arm of chromosome 11. Sequencing the insulin gene very quickly led to the large-scale production and availability of human insulin and in turn to the development of insulin analogues that are at the forefront of treatment for T1DM. It prompted a rush to understand how the insulin gene was regulated, and to the discovery of the transcription factors (TFs) involved in this process. These TFs play a major role in cell fate decisions in the developing pancreas, and as these processes became understood in detail, their use was vital in generating alternative sources of islets from pluripotent cells.

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https://doi.org/10.15277/bjd.2022.371

This article is written from a personal perspective and should not be taken as an extensive review of reprogramming. At an early stage my lab became interested in how the human insulin gene was regulated,[5] and our work contributed to the identification of a key transcription involved,[6] and the structure of the adjacent hypervariable region.[7] We were keen to exploit the clinical applications of these findings and the route took us sequentially through gene therapy, replenishable supplies of islets from embryonic stem cells, and reprogramming. There now follows a brief account of our experience in each of these areas.

Gene therapy for diabetes

For us gene therapy for diabetes involved the administration of an insulin gene DNA construct to patients. The idea was that the injected DNA would be taken up by cells and transcribed and translated into protein that would be secreted constitutively into the blood stream. The assumption was that expression would be retained for some period of time and would obviate the need for daily insulin injections. There were three main challenges.

The first was which cell to select for expression of the exogenous gene and how to promote efficient uptake into this cell type. At that time (the 1990s) in vivo gene therapy had been directed at several monogenetic disorders and mostly involved viral-mediated gene delivery. The field underwent a major setback in 1999, however, with the tragic death, following administration of a normal gene within an adenoviral vector, of a patient (Jesse Gelsinger) who had been recruited to a safety trial for gene therapy for ornithine transcarbamylase (OTC) deficiency. The news that an experimental treatment had killed a basically healthy volunteer represented a major setback for the field of gene therapy. After many years the field eventually recovered, and gene therapy is now an extremely attractive area of medicine.

For these reasons our approach at that time was to avoid the use of viral vectors and to use instead naked DNA, which was injected directly into the muscle. Muscle was selected as the most amenable site of injection. The uptake of DNA was very inefficient and sowed doubts in our mind as to the viability of this approach.[8-10] However, there is a strong argument for revisiting this approach using gene delivery systems such as advanced adenoviral constructs and RNA-mediated systems as developed for the Covid-19 vaccination programmes.

The second challenge was related to the processing of proinsulin to insulin that in the pancreatic beta cells involves two proteolytic enzymes (PC2 and PC1/3). The problem was that non-neuroendocrine cells lack PC2 and PC1/3. This was surmounted by engineered site-directed mutagenesis of the proinsulin cleavage site between the B-chain/C-peptide junction (Arg-Arg) and the C-peptide/A chain junction (Lys-Arg) to be recognised by furin, an endoprotease that is expressed in muscle and a wide variety of other cell types. When transfected into a muscle line this furin-cleavable construct was efficiently processed to mature insulin and expressed at the same level as wild type proinsulin.[11] This suggests that furin-cleavable (pro)insulin constructs will work in a variety of cell types in the context of gene therapy.

The third problem was how to regulate secretion of insulin from the transduced muscle cells. In the beta cell insulin is stored in secretory granules and released in response to changes in circulating blood glucose levels. Muscle lacks this regulated secretory pathway and would constitutively secrete insulin as it was synthesised. If secreted at very low levels, as expected, this may not be problematic, and a very low background level of insulin in T1DM, and indeed T2DM, patients might be of therapeutic value. However, ideally one would prefer some regulation of insulin release in response to glucose. Importantly, the insulin gene responds to glucose stimulation via pathways that are not well understood although the major regulatory sequences and TFs have been identified. Because these TFs are mostly beta cell-specific, glucose-responsive regulatory sequences within the insulin promoter would be unlikely to work in muscle. Fortunately, the L-type pyruvate kinase (PK) gene is regulated by glucose through known DNA sequences that would function in muscle. We therefore constructed a hybrid gene contained the PK regulatory sequences upstream of the insulin (engineered for cleavage) coding sequences. This worked extremely well (unpublished data); improvements might involve a global screen of DNA libraries for sequences that are glucose-responsive in muscle.

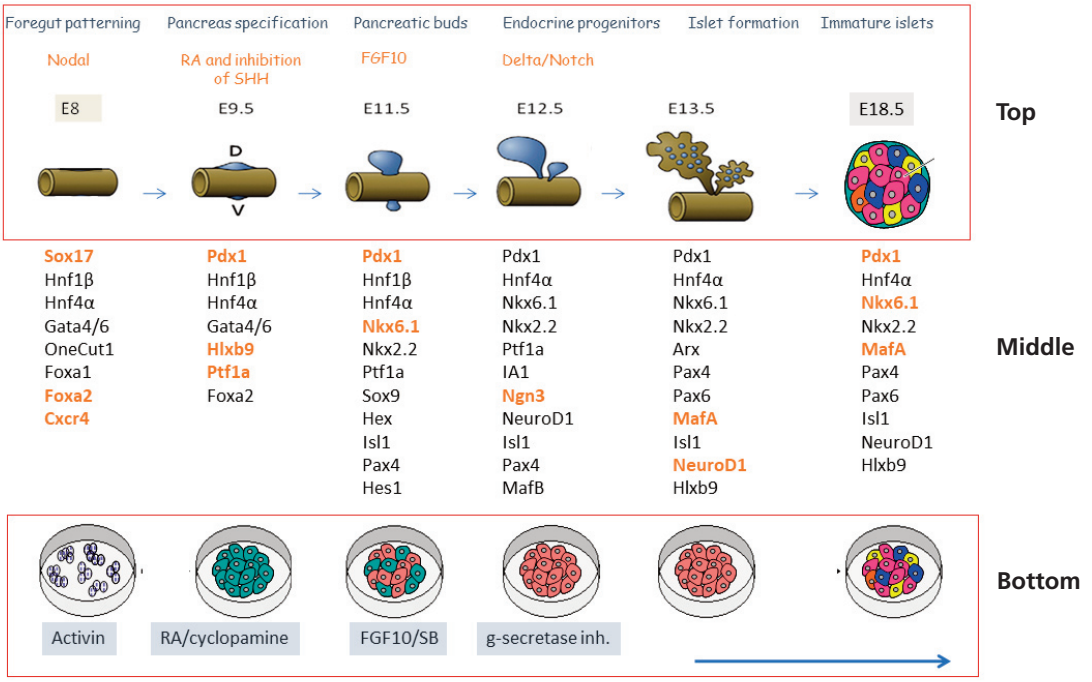
In conclusion, despite our previous reservations about safety issues of viral transduction and low levels of expression, *in vivo* gene therapy (muscle directed) has without doubt a role to play in the treatment of T1DM and T2DM.

A replenishable supply of islets from embryonic stem cells

Cadaveric islet transplantation, based on the Edmonton protocol,[12] is an NHS- funded clinical service in the UK. It can both reduce the frequency of severe hypoglycaemic events (SHE) and improve hypoglycaemic awareness (IHA) in more than 90% of patients.[13] However, widespread application is limited by the lack of suitable donor pancreases, with only 30-40 transplants carried out each year in the UK. The problem is compounded by the fact that the procedure typically requires at least two islet infusions from multiple donors. As a result, transplants are targeted at patients suffering from SHE and IHA who struggle to control their diabetes with conventional insulin therapy. These patients represent 10% (SHE) and 5% (IHA) of the total T1DM population (350,000 in the UK) and the available transplants go nowhere near to meeting this target.

One way of addressing this unmet demand might be to grow islets in culture. However, this has proved virtually impossible. Embryonic stem cells on the other hand grow well (expanded) in culture, whilst maintaining pluripotency, and theoretically can be induced to differentiate towards any cell type. In the case of pancreatic islets, the approach is to recapitulate in a culture dish the events that occur in the developing pancreas.[14] The overall strategy that we employed is shown in Figure 1. The top panel is a simple schematic showing stages in the developing mouse pancreas. (The human pancreas follows similar pathways, although over a much-extended time scale.) By embryonic day 8 (E8) the primitive endodermal gut tube has formed. At around E9.5 the two lobes of the pancreas grow out from either side of the gut; over a period of

Figure 1. Schematic depicting the approach towards generating islet-like cells from embryonic stems cells (ESCs) and induced pluripotent cells (iPSCs)



The **top panel** shows the events that occur in the developing pancreas with some of the important factors that drive these processes. The strategy (**bottom panel**) is to mimic these events in a culture dish using ESCs or iPSCs as starting material. Progress is monitored by measuring transcription factors (**middle panel**) by RT/PCR and immunocytochemistry. More sophisticated protocols have been developed, whereby the cells are treated for an extended period of time (30-35 days) resulting in fully functional mature beta cells

days these anlages expand and eventually the two lobes merge. Islet formation commences at around E13.5 with immature islets forming around E18.5, just before birth. Further maturation occurs in the days after birth. These events are controlled by several growth factors that include Nodal, retinoic acid (RA), sonic hedgehog (SHH), FGF and delta/notch signalling, as shown.

The bottom panel depicts how these events can be mimicked in a culture dish. Embryonic stem cells are first cultured in high doses of Activin A to induce formation of primitive endoderm. The cells are then sequentially treated with RA/cyclopamine, FGF, an SB reagent that inhibits liver formation, and a gamma secretase inhibitor. Progress along this pathway can be monitored by measuring by RT/PCR and immunocytochemistry the transcription factors that are expressed at each stage (middle panel). These can be viewed as a barcode, and the closer one gets to the complete barcode the better the outcome.

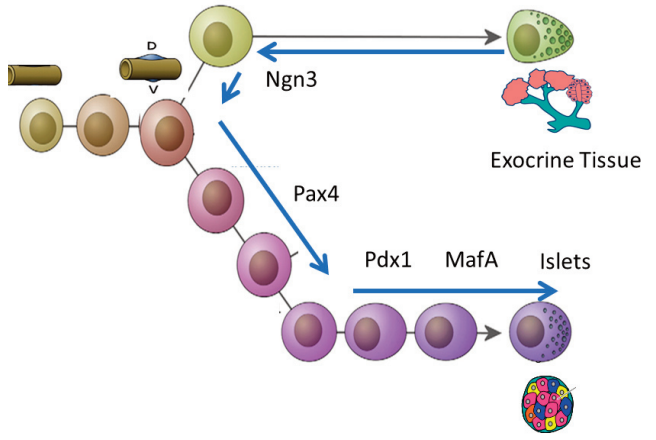
This is typical of the protocols that were developed in our laboratory. In more recent years related protocols have been extended beyond 18 days to generate fully functional islets that exhibit a secretory response to glucose and express levels of insulin close to those seen in adult human islets.[15-17] A biotech company, ViaCyte, has developed methods for encapsulating human ESC-derived islets and human phase 1 safety trials have been underway for several years. For reasons related to the technical difficulties in differentiating human ES cells over extended periods of time and the competition from the large Biotech ventures we terminated this project and moved towards reprogramming.

Reprogramming pancreatic tissue

The reprogramming project arose as a collaboration between our laboratory in Aberdeen and the Scottish Islet Transplantation Centre (SITC), which is located 205 km away in Edinburgh. The SITC was established in 2007, and the first islet transplant into humans took place in 2011. Currently the programme performs around 20-30 transplants per year (it is the major UK centre). The transplant recipients are immunosuppressed and at present the treatment is restricted to adults with SHE and IHA, as described above.

We were involved at an early stage in discussions as to how the unmet demand could be achieved and came up with reprogramming as an alternative to generating islets from ES cells. Islets of Langerhans represent about 2% of the total cell content of the pancreas. The remaining 98% of the tissue comprises acinar cells that produce hydrolytic enzymes and ductal cells that collect these enzymes and direct them to the small intestine. Following extraction of the islets, the left-over exocrine tissue is normally discarded in a manner sensitive to their human origins. Our idea was that the discarded exocrine tissue could be converted into functional islets. Theoretically, if 100% efficient this would generate 50 transplantable units per donor pancreas, which would have a huge

Figure 2. Ball diagram depicting the overall strategy towards reprogramming pancreatic exocrine tissue towards functional islets



During development of the pancreas, around the time when the dorsal and ventral (D and V) anlages appear from the primitive gut tube, there arises a cell type that give rise to both exocrine and endocrine cells. Based on our knowledge of the transcription factors that control these events we predicted that Ngn3 would drive exocrine towards this progenitor cell type and that a combination of Pax4, Pdx1 and MafA would drive differentiation towards islets. This would be further facilitated by inducing an intermediate EMT (iEMT) stage that might exhibit increased plasticity

impact on the roll-out of islet transplantation. Of course, expected efficiency would be nowhere near this but even 10 or so transplantable units per donor pancreas would be a huge improvement on current numbers.

We had been toying with the idea of generating islets from other mature cell types throughout the 1990s, but the concept of transdifferentiation, i.e. converting one mature cell type into another, although championed by some,[18] was met by a great deal of scepticism. Things changed in 2006 with the elegant studies of Yamanaka,[19] who showed that fibroblasts could be converted into pluripotent stem cells (iPSCs) using a combination of four transcription factors, namely Oct4, Sox2, Klf4 and c-Myc (OSKM). Our approach was also to use transcription factors, in this case those that played a pivotal role in cell determination in the developing pancreas. At an early stage in pancreatic development there appears a progenitor cell type that gives rise to the exocrine, ductal and endocrine cells. Which route this cell takes is dependent on the expression of a set of transcription factors that include, amongst others, the keys players Pdx-1, Maf-A, Ngn-3 and Pax-4 (PMNPx). Our overall strategy (Figure 2) was that Ngn-3 would drive the acinar cell population towards this progenitor cell type and that a combination of Pdx-1, Maf-A and Pax-4 would drive the resultant cell population towards a beta cell phenotype. As the project developed, we tested a combination of other pancreatic transcription factors, but none worked better than the PMNPx cocktail.

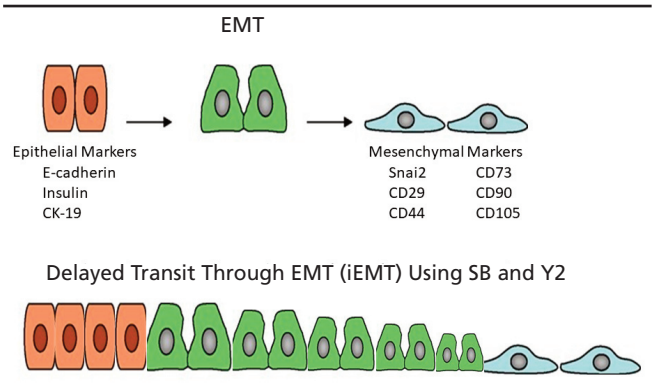
There now follows an account of how the final protocol was developed. Following islet isolation, and with appropriate ethical approval, the low-purity exocrine fraction was transported from Edinburgh to our laboratory in Aberdeen. The digested extract contained clusters of cells measuring between 50-200 microns. Immunofluorescence microscopy showed that the clusters contained predominantly amylase-staining acinar cells and some cytokeratin-19 (CK-19)-staining ductal cells. The presence of islets, as monitored by dithizone staining, routinely represented less than 1% of the total tissue. The exocrine-enriched fraction was then cryopreserved.

For reprogramming the frozen clusters were slowly thawed and plated on tissue culture plates. Over a period of 72h the clusters attached to the dish and an outgrowth of cells with the characteristic appearance of fibroblasts began to populate the empty space on the dish. Initially the outgrowth contained amylase and CK-19 positive cells but this staining, and mRNA levels for these proteins as measured by RT/PCR, diminished with time and were undetectable after 72h in culture. The fibroblast-like cell population could be passaged, providing a considerable expansion in cell number. Routinely the cells were passaged 6-8 times without noticeable changes in the cell population. Passaging beyond eight times would likely lead to culture-dependent clones within such a rapidly expanding population. The resultant cell population exhibited many of the properties of mesenchymal stromal cells (MSCs) in terms of cell surface markers (CD90, CD107 and CD73), and in keeping with the properties of MSCs they could also be induced to differentiate towards osteoblast, chondrocyte and adipocyte lineages under appropriate culture conditions. We showed by genetic lineage tracing that these MSC-like cells were derived from the epithelial clusters by a process of dedifferentiation similar to the epithelial to mesenchymal transition (EMT).[20]

Clearly in order to generate beta cells we would need to convert these MSC-like cells back to epithelial cells, i.e. induce a mesenchymal to epithelial transition (MET). This was effected by culturing the cells in media supplemented with 5-aza-2'-deoxycytidine, sodium butyrate, SB431542 and Y27632 for three days. EMT and its converse MET is a gradual process in which the cells pass through intermediate stages (Figure 3 adapted from[21]). Our rationale was that, even though the cells failed to transit completely to epithelial cells, the intermediate stages would exhibit a degree of plasticity which would enhance the effects of the exogenous pancreatic transcription factors. We had shown previously that KLF-4, one of the Yamanaka factors, could achieve a similar effect in reversing the process i.e. effect a mesenchymal to epithelial transition (MET).[22]

We also attempted to determine if a specific MSC had some memory and hence specific properties related to its origin. We did this by isolating MSCs, by fluorescence-activated cell sorting (FACS), that were genetically marked with the fluorescent marker dsRED. In this system the initial plated cultures that were amylase-positive or islet-derived cultures that were insulin-positive were genetically tagged by introducing by lentiviral transduction a DNA construct in which expression of the fluorescent marker dSRED was under the control of the amylase or insulin promoter. Over a few passages the resultant MSC-like cells continued to express dsREd, thus identifying their cell of origin. However, the results comparing MSCs that were originally Beta (INSdsRED) or acinar (AMYLdsREd) cells were inconclusive. It would be important to resolve this issue, since it is possible that the culture conditions, including properties of the

Figure 3. Generating an intermediate epithelial to mesenchymal transition (iEMT) stage that would enhance cell plasticity



The top panel shows the stages in the EMT, with appropriate markers. The lower panel shows that by using the reagents SB and Y2 we could delay transit through the EMT thus generating an iEMT stage that would exhibit increased plasticity and enhance the effects of the transcription factors

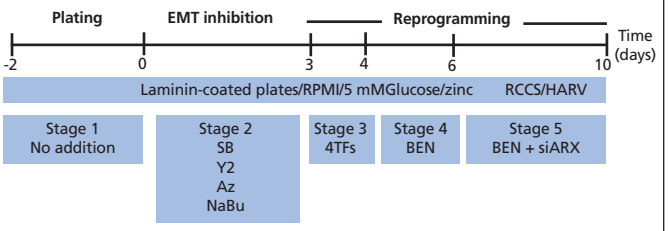
plastic dishes, the coating used and the culture media, may be more important than the tissue source of the MSCs. The implication is that tissues other than pancreas could be used in reprogramming if our hypothesis regarding the plasticity of the intermediate EMT (iEMT) stage were correct.

After three days the transcription factors (PMNPx) were introduced into the cells using replication-deficient adenovirus-mediated transduction. Initially we used four separate viruses, but later we used two viruses, one harbouring Pdx-1 and Maf-A and another Pax-4 and Ngn-3. At that time there were no safety concerns with the ex vivo use of adenoviruses. Viruses containing three transcription factors were less efficient, in that the order in which the TFs were placed relative to the gene regulatory sequences affected the expression levels observed within the cells. At this point the cells were cultured in serum free media supplemented with betacellulin, exendin-4 and nicotinamide, factors that are known to promote beta cell differentiation. These factors alone had very little effect on the expression of insulin as measured by RT/PCR, but significantly enhanced the effect of the exogenous TFs.

The final stages of the differentiation protocol involved treating the cells with an siRNA targeting the transcription Arx which favours differentiation towards glucagon-expressing alpha cells.[23,24] The inclusion of zinc enhanced insulin production, presumably through its requirement for the formation of insulin hexamers. The final stage involved transferring the cells from the culture dishes to a rotating cell culture system (HARV).[25] This step facilitated cell aggregation under effectively microgravity conditions and the formation of islet-like structures.

The final protocol (Figure 4) takes around 12 days and involves plating the isolated human exocrine tissue on laminin-coated plates and culturing in media containing 2.5 mM glucose.[26] Initially, the EMT is partially inhibited using Rho kinase and TGF-β inhibitors in combination with methyltransferase and histone deacetylase inhibitors. Generating this iEMT cell population is particularly important. This is followed by transduction with the exogenous TFs, Pdx1, MafA, Ngn3 and Pax4 and further culture in media containing betacellulin, nicotinamide and exendin-4. Inhibition of endogenous Arx by siRNA is performed towards the final stages of the protocol. The final stage involves culture in a rotating vessel to facilitate aggregation and formation of islet-like structures.

Figure 4. Protocol for generating functional islet-like cells from human exocrine clusters



Details of the protocol are provided in the text

The final product was around 40% endocrine, with a mixture of cells expressing either insulin or glucagon (but not both). The remaining cells were pancreatic MSCs which have the added advantage in terms of the function of the transplanted tissue. The cells were stable as evidenced by the prolonged appearance of human C-peptide in the blood of transplanted mice and the morphology of the transplanted aggregates at periods up to 100 days after transplantation. The reprogrammed cells were responsive to glucose under static incubation conditions and they exhibited a biphasic response to glucose in a perfusion configuration.

In summary, the reprogrammed cells met all the important criteria that would be needed for clinical applications. They expressed fully processed insulin, i.e. they efficiently converted proinsulin to insulin, at therapeutic levels. They efficiently stored, processed and secreted insulin in response to glucose and other nutrients, in a manner similar to adult human islets. They normalised blood glucose levels in an appropriate diabetic animal model. The cells were phenotypically stable. We estimated that one transplantable unit would contain 1-2 billion cells. With efficient expansion of the MSC-like stage each donor pancreas could provide around 10 transplantable units.

We then moved towards taking the project into the clinic.[27] The first stage was to undertake a rigorous assessment of the challenges involved, with a number of go, no-go milestones. The protocol was able to pass an economic viability assessment; it was deemed amenable to scale up under GMP (Good Manufacturing Practices), and no regulatory or licensing issues were identified. However, it was clear that before the project could be taken forward into expensive animal safety and toxicity studies and from there to clinical trials it would require further protocol development and refinement. The major problem was related to batch-to-batch differences in reprogramming efficiency such that it was almost impossible to write rigid standard operation procedures. These differences could in part be related to differences in the donor material

but it was impossible to investigate this since there were clear differences in outcome that could be directly attributed to the protocol.

One potential problem was the distance (205 km) between the islet isolation centre and the reprogramming laboratory. On a good day we would receive the pancreatic tissue within eight hours or so, while on other occasions it was clear that the tissue could be 48h and sometimes 72h old. Regardless of the age of the tissue it was always in good shape, surprisingly, as measured by appearance under the microscope and ability to attach to the culture dish and expand in culture. However, ideally one would place the reprogramming lab adjacent to the isolation lab, and this could easily be arranged. This would also negate the requirement for cryopreserving the clusters and facilitate maintenance of GMP conditions. The major problem, however, was in allowing the clusters to attach to culture dishes and undergo EMT. We felt at the time that delaying EMT through intermediate stages (iEMTs) that might exhibit favourable plasticity would enhance the effects of the TFs, whilst also allowing expansion of the cell population. In fact, we initiated a screening programme for small molecules that would better achieve this goal, with some encouraging hits. An alternative approach might be to maintain the clusters in a bioreactor and optimise the conditions for efficient adenoviral transduction in a 3-D configuration. This would circumvent the potentially unnecessary stages of inducing and then reversing the EMT but would preclude any cell expansion stage. With increased efficiencies and reduced losses it may well be possible to generate a higher yield of transplantable units with enhanced islet-like characteristics.

In summary, our protocol generates β-cells that share many of the properties of adult endogenous β-cells and compare well with surrogate β-cells generated from human embryonic stem cells. Our approach has the advantage that the cells are not at any stage pluripotent, which has important safety considerations. In addition, the reprogramming protocol is relatively simple, cost-effective, adaptable to clinical grade good manufacturing (GMP) conditions, and at 12 days is significantly shorter than the time required to generate fully functional β-like cells from hESCs. We estimate that around 3-5 x 10^8 reprogrammed cells would have a therapeutic effect if transplanted into patients with diabetes; thus one donor pancreas could provide numerous (~10-12) islet grafts. For these reasons we believe that modifications to the protocol as described could lead to a viable cell therapeutic for the treatment of diabetes. One hundred years after the first treatment with a pancreatic extract enriched in insulin we could soon be moving towards administration of insulin via transplantation of reprogrammed alternative cell types.

Summary

The discovery of insulin more than 100 years ago was a team effort, involving a physiologist (MacLeod), a surgeon (Banting), a medical intern (Best) and a chemist (Collip) and very quickly the recruitment of the might of the pharmaceutical industry. Clearly, since the discovery of insulin, there has been a need for new therapeutic approaches that will obviate the need for multiple daily injections and give better metabolic control. Here we have described advances in cell and gene therapy and how ES-derived islet cells are already in clinical trials. It is clear that progress will continue and as experimentalists we should always have a view on how these therapies should be taken to the clinic. It is important to assemble the team as the therapies are being developed rather than wait.

Key messages

- There has been much recent progress in cell and gene therapy to address diabetes
- Encapsulated islets derived from human stem cells are undergoing clinical trials
- Reprogrammed islets from human pancreatic tissue may be taken forward into preclinical studies
- Gene therapy may involve injecting nucleic acids encoding insulin into muscle

Conflict of interest None.

Funding Supported by grants from Diabetes UK (gene therapy), JDRF (ES-derived islets) and Wellcome Trust, MRC and Cell and Gene Therapy Catapult (reprogramming).

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