[Frontiers in Bioscience 19, 126-138, January 1, 2014]

Scaffolds for islets and stem cells differentiated into insulin-secreting cells

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1. ABSTRACT

Embryonic/pluripotent stem cells offer the possibility of an unlimited source of cells to be differentiated into beta cells. This requires differentiating the stem cells into pancreatic progenitors by tissue culture, and then transplanting into recipients for the final stages of development into mature beta-cells. Exposing embryonic stem cells seeded onto laminin coated PLGA scaffolds to biochemical cues resulted in enhanced expression of definitive endoderm markers compared to those differentiated on 2D monolayers. The production of tissue specific cells from stem cells can be scaled up using bioreactor cultures. To apply human stem cell derived islet progenitors in a clinical setting, one must first overcome the problem of immune rejection. Immuno-isolating the cells using microencapsulation provides one possible solution. Coating scaffolds with an anti-inflammatory agent could be an effective means of reducing the inflammatory process that results in pericapsular fibrosis and necrosis of the encapsulated cells. This review summarizes the above issues and describes how 3D scaffolds seeded with stem cells and/or pancreatic progenitors may provide a benefit to achieving normalization of blood glucose levels.

2. INTRODUCTION

The transplantation of islets isolated from donor human pancreases is currently the main cellular therapy available for people with type 1 diabetes mellitus (T1DM). However, the supply of islets is vastly less than the number of potential recipients. Moreover, several pancreases are often required to be able to isolate a sufficient number of islets to achieve normalization of blood glucose levels (euglycaemia). Whilst euglycaemia is usually achieved in recipients of human islets, that state is not maintained over the ensuing years after transplantation, with most requiring re-introduction of insulin administration within 2-3 years to control blood glucose levels (1). To improve clinical results requires transplantation of a greater number of islets with better functional capability and/or at a site where instant blood mediated immune response does not result in loss of half the islets within minutes of their infusion (2). Greater efficiency of islet isolation can be achieved by enhancing the expertise of operators and by improving the activity of the enzyme used to separate the islets from the exocrine tissue. Improving the functional capacity of islets includes administering agents to the recipient to enhance insulin release, for example, exenatide; or reducing the adverse

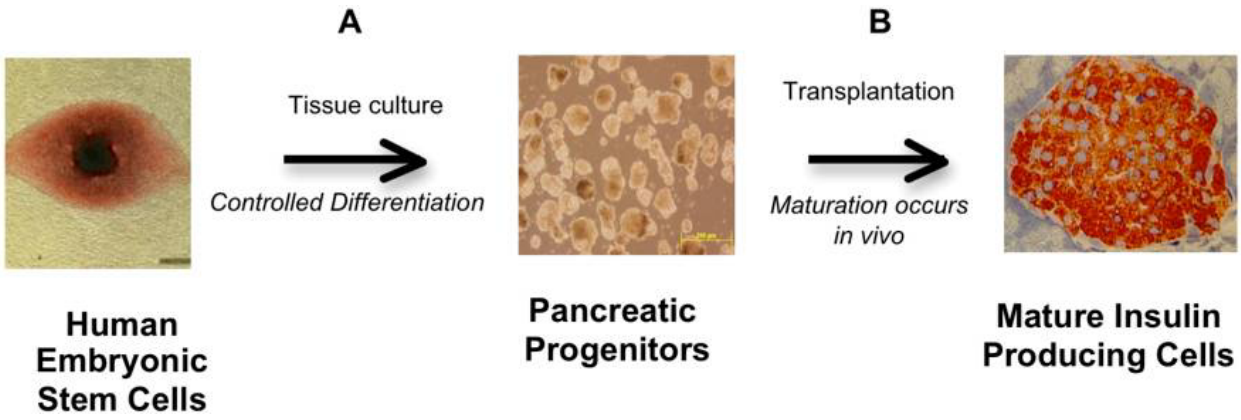

Figure 1. Two step procedure to produce mature glucose responsive insulin producing cells from hESC. A. *In vitro* differentiation of hESC to PP, and B. *In vivo* differentiation of PP to mature beta cells.

effect of inflammatory cytokines, for example, with a blocker of the receptor for tumour necrosis factor alpha, infliximab, and diminishing the effect of instant blood mediated immune response, for example, by coating the islets with a heparin conjugate (3).

With the assistance of bioengineers, a new method of improving the functional capacity of islets is emerging. This requires the seeding of islets onto biodegradable three-dimensional (3D) scaffolds. The 3D nature of the device mimics the physiological milieu of the pancreas (4) and this should enhance function. A second reason for the beneficial effect of 3D scaffolds is that they can be engineered to vascularise readily (5, 6), meaning greater oxygenation of cells seeded on it. Since ß-cells function better in high rather than low concentrations of oxygen, greater efficiency should be achieved with islets seeded onto the scaffolds. In this review we first discuss alternative sources of ß-cell surrogates derived from stem cells that could be used for T1DM cell replacement therapy. We then focus on the possibility of utilising 3D biodegradable scaffolds as vehicles to improve the outcomes of this type of therapy.

3. ALTERNATIVE SOURCE OF BETA CELLS

The supply of human pancreases from which human islets are obtained for therapeutic purposes is limited. In Australia, for example, there were 35 cadaveric pancreases donated in 2011, and of these 26 were used for whole organ pancreas transplants, leaving only 9 for possible islet isolation (7). Since sufficient numbers of viable islets suitable for therapeutic use are isolated from fewer than half the available pancreases, the number of Australians with T1DM transplanted with islets was very small. And yet the number of people in Australia with type T1DM is 149,000 (8). This disparity between availability of islets and potential recipients is the same across the globe. There are at least 1,119 people with T1DM who have been transplanted with human islets (9), but the number of people with this type of diabetes is estimated to be 18.3 million [estimate based on 5% of total world diabetes population of 366 million] (10).

Stem cells, especially pluripotent cells derived from embryos, are a potential source of surrogate beta cells to replace cadaveric islets (Figure 1). Human embryonic stem cells (hESC) are available in unlimited numbers, and can be converted into pancreatic progenitors (PP) by a series of culture steps over a period of 2 weeks (11-15). The steps in their conversion follow ontogeny with the first step being formation of definitive endoderm, and the second foregut endoderm. Thereafter, PP are created, characterized by expression of the key pancreatic transcription factors PDX1 and SOX9.

Ability to convert PP to mature beta cells is achieved by transplanting the PP into immunodeficient mice rendered diabetic with streptozotocin (13). The time taken for differentiation into beta cells and their subsequent maturation into glucose-responsive cells is 2-3 months. This is a similar process to what occurs when PP from human fetal pancreas are transplanted into mice, as was first described in the 1980s (16, 17). As yet, there is no reliable *in vitro* method for the differentiation of hESC/PP cells into fully functional mature ß-cell surrogates. Therefore, currently it is necessary to allow for *in vivo* differentiation in order to derive glucose responsive ß-cell surrogates. Consequently PP rather than mature beta cells are currently the preferred cell type for transplantation into diabetic recipients. When addressing the issue of transplanting cells within scaffolds, the type of cell to be transplanted should be taken into account as this has implications for the selection of scaffold material and also scaffold design. Specific scaffolds can then be tailored to promote both the maturation and development of the transplanted PP as well as encouraging the ingrowth of supporting tissue structures including host endothelial cells.

Induced pluripotent stem cells (iPSC) could be an alternative source of pluripotent stem cells to hESC, and are likely to be preferred by those who view the use of hESC as ethically unacceptable; however, there is some doubt about this possibility because of their immunogenicity (18). iPSC generated from fibroblasts are capable of differentiating *in vitro*, in a manner similarto

Table 1. History of the development of cell therapies for insulin-dependent diabetes[1]

Year	Event
ISLETS	
1965	Islets isolated from a guinea pig pancreas
1967	Islets isolated from a rat pancreas
1971	Islets isolated from a human pancreas
1972	Normalization of blood glucose levels in isografted diabetic rats
1977	Islets transplanted into a diabetic human
1989	Normalization of blood glucose levels in a type 1 diabetic human with allografted islets
2000	Sustained normalization of blood glucose levels in a series of people with type 1 diabetes that received allografted islets
STEM CELLS	
1998	Human embryonic stem cell line created
2003	Autologous mesenchymal stem cells transplanted into a person with type 1 diabetes
2008	Normalization of blood glucose levels in diabetic mice transplanted with pancreatic progenitors differentiated from human embryonic stem cells
2008	Allogeneic mesenchymal stem cells transplanted into a person with recent onset type 1 diabetes
2009	Autologous cord blood cells transplanted into a child with type 1 diabetes

[1]From the 1960's when immunosuppressive therapy began to become available. The year quoted is the first time the event is thought to have occurred

ESC, and forming definitive endoderm, and subsequently insulin-producing cells. Approximately 10% of these cells were positive for insulin and c-peptide was detected at elevated levels upon exposure to various stimuli, but not glucose (19). However, transplantation into diabetic mice with these cells results in lowering of blood glucose levels (20).

In addition to these pluripotent stem cells, there are multipotent precursor cells that have been identified in both mouse and human pancreas. Whilst they are capable of differentiating into beta cells (21), their numbers are too small to suggest therapeutic usefulness.

4. HISTORICAL PERSPECTIVE

The possibility of using hESC as a therapy for insulin-dependent diabetes is a relatively recent phenomenon. It began a decade ago, not long after the isolation of the first hESC line in Madison, Wisconsin (22). Initial attempts to convert ESC into glucose-responsive insulin-producing cells followed a neurological developmental route via the protein NESTIN (23), rather than the endodermal ontological route, and were unsuccessful. Several years later, the route changed to following the pathway for pancreatic development that occurs in the fetus, with the formation of definitive endoderm from hESC (24) as the first significant stage of differentiation. Clinical trials with hESC for treatment of T1DM are not yet ready for the clinic, but may be by 2013, with the Californian company Viacyte Inc. leading the way. The possibility of using hESC as a diabetes therapy has been heightened by the commencement of first-in-man trials in October 2010 with cells derived from hESC, for treatment of acute spinal cord injuries by another Californian company Geron Inc.. This initial trial with hESC occurred just 12 years after the isolation of the first hESC line. Obtaining regulatory approval for this from the FDA, however, was fraught with difficulty. Since then, a 2nd Californian company, Advanced Cell Technologies, obtained approval from the FDA to use retinal pigment epithelial cells derived from hESC for treatment of macular degeneration.

In trying to look into the future about how long it is likely to take for PP derived from hESC to yield positive outcomes in diabetic humans, it is helpful to look at the history of islet isolation and transplantation (see Table 1 for a summary). It was in 1965 when islets were first isolated from the pancreas of a guinea pig (25) and in 1971 from a human pancreas (26). The first clinical trial with human islets, however, was not until 1977 (27), and it took a further 12 years before the first human recipient was able to cease endogenous insulin administration (28), albeit briefly. It was not until 2000, however, before the success rate improved dramatically, mainly because of an alteration in immunosuppressive protocol being administered to recipients (29). This was called the Edmonton protocol, because of the city in Canada where the trial was conducted. If these time frames relevant to the therapeutic development of human islets are translated for the development of hESC therapeutics, it will not be until 2016 before the first person will cease insulin after being transplanted with cells derived from hESC, and 2027 before consistent success is achieved. It is recognized, however, that future dates are very speculative.

5. 3D BIODEGRADABLE SCAFFOLDS

Key issues in accelerating the pathway to the clinic will be optimizing the rate of formation of mature beta cells from hESC. The traditional approach is to culture the cells in a 2D environment. Attaching hESC on 3D scaffolds to differentiate them towards PP may accelerate this process, just as attaching human islets on 3D scaffolds improves the outcome of the islets, at least in rodents (30-33), as compared to grafting unseeded islets. The 3D matrices are capable of promoting differentiation and enable the formation of more complex tissues (4, 6, 34-36) that are histologically similar to tissues found *in vivo* (6). It is argued that the 3D environment created by the scaffolds can more closely mimic the *in vivo* microenvironment of the developing tissues (4). The porous structure of a scaffold enables nutrient access for the cells from the centre of the scaffold and at the same time creates the 3D physical structure that has been shown to be beneficial for cellular differentiation (37). Additionally, growing cells on 3D scaffolds rather than as 2D monolayers can encourage 3D structure formation, cellular migration and graft vascularisation (5, 6).

Numerous different materials are available for scaffold manufacture including polyesters polylactic-co-glycolic acid (PLGA) and poly-L-lactide acid, polyurethanes, polyanhydrides, naturally occurring proteins (collagen, elastin, fibrin), polysaccharides (hyaluronic acid)(38) and decellularized mouse pancreas (Table 2). Each individual material has characteristics that may suit

Table 2. Scaffolds currently available[1]

Matrices	Cell types grown	References
Synthetic polylactic-co-glycolic acid	islets	(30, 32, 33, 40, 49)
	cardiomyocytes	(50)
	human embryonic stem cells	(4, 42)
	neuronal cells	(35)
	skeletal muscle	(6)
poly-L-lactide acid	islets	(31)
poly D,L -lactic acid	neuronal cells	(96)
chitosan	islets	(97)
polyurethane	cartilage	(98)
polyanhydride	bone	(99)
parylene	retinal pigment epithelium	(100)
Natural collagen	islets, hepatocytes	(31, 34)
elastin	lung	(39)
fibrin	mesenchymal stem cells	(101)
hyaluronic acid	islets, hepatocytes	(102)
decellularized mouse pancreas	insulin-producing cells	(41)

[1]Some examples of the various types of cells that are grown on scaffolds are provided

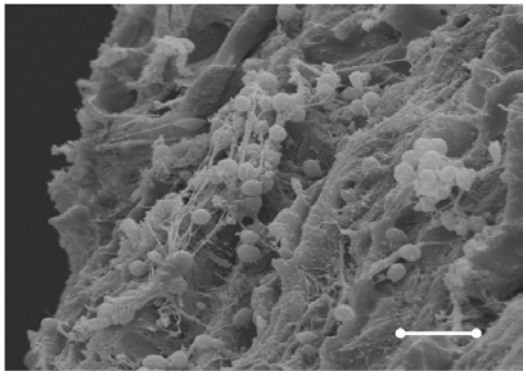

Figure 2. Scanning electron microscopy of PLGA scaffold seeded with human embryonic stem cells. Fabrication techniques enable the manufacture of scaffolds to the desired size. The pore sizes in this scaffold are 125 – 250 μm.

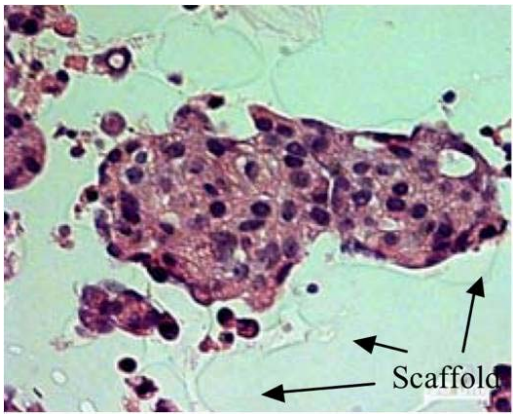

Figure 3. Haematoxylin and eosin stain of human islets seeded onto PLGA scaffolds.

certain transplantation procedures (Figure 2). Elastin, for example, may be useful in cell replacement therapies where tissue that undergoes substantial deformation such as the heart or lung (39). For replacement of islet tissue using PP the geometry and components of the islet should be used as a guide for design and development of appropriate scaffolds. When choosing an appropriate material, consideration should be given to its degradation time and the degradation products.

5.1. Islets

The beneficial effect of mouse islets seeded on to biodegradable scaffolds made of PLGA was shown by Lowe and colleagues at North Western University in Chicago (30). They demonstrated that a minimal mass of 125 islets from C57BL6 mice, seeded onto the 3D scaffold, composed of lactide:glycolide of 75: 25 and with a porosity of > 94%, normalized blood glucose levels of recipient syngeneic diabetic mice when placed in the epididymal fat pad. In contrast, euglycaemia could not be achieved when the same number of islets was transplanted without a scaffold. Moreover, the islets seeded onto the scaffolds functioned more efficiently. Whilst normoglycaemia was achieved when 175 islets were transplanted either on or off scaffolds, blood glucose levels became normal faster with the seeded islets. As well, the rate of clearance of glucose, as shown in a glucose tolerance test, was significantly greater with islets on scaffolds.

Very recently, similar results have been achieved with human islets seeded on similar scaffolds. Figure 3 shows an islet attached to a PLGA scaffold. Human islets seeded onto scaffolds of this type and coated with extracellular matrix (collagen I, collagen IV and fibronectin) survived longer *in vitro* than if maintained in a 2D system (31). The composition of these scaffolds was 85:15 (lactide:glycolide), their porosity 67%, pore size 351 μm and strand thickness 107 μm. Moreover, the efficiency of human islets to normalize blood glucose levels of diabetic recipient mice was enhanced when they were seeded on to scaffolds prior to transplantation (32).

5.2. Stem cells and progenitors

There is only one publication we are aware of describing the attachment of insulin-precursor cells to PLGA scaffolds and their subsequent transplantation into immunodeficient mice (40). hESC were differentiated *in vitro* from nestin-positive progenitor cells and then exposed to exenatide to encourage insulin production. Mature beta cells were not produced as the cells were not glucose responsive. These cells were seeded onto PLGA scaffolds (glycolic acid:lactic acid = 90:10) of dimensions 0.5 x 0.5 x 0.5 mm^3 at a density of 0.8 -2.4 x 10^7 cells per scaffold. Porosity was 90% and pore size 100 – 300 μm. The cell-scaffold complexes were cultured for 3-5 days before being implanted subcutaneously into immunodeficient streptozotocin diabetic mice. As a control, non-seeded cells were grafted beneath the renal capsule of the mice. There was some evidence of insulin production in both groups of grafted mice, but no advantage was demonstrated with cells seeded onto the scaffolds.

The transplantation of PP derived from hESC within Gelfoam scaffolds has been reported. The authors stated that cells seeded within scaffolds performed as well as cells that were transplanted within free aggregates as measured by achievement of euglycaemia (13). However, there was no further analysis comparing other important parameters that may benefit from scaffold utilisation including revascularisation of the graft or maintenance of cell aggregate structure.

In addition to the above, a group in Pittsburgh has recently reported differentiating hESC to PP on a scaffold of decellularized murine pancreas. The microstructure of the acellular pancreas was preserved as were its extracellular matrix proteins (see below). qPCR of hESC differentiated on this scaffold showed a 53-fold increase in PDX-1 and a 93-fold increase in INSULIN expression compared to cells differentiated on Matrigel (41).

6. EXTRACELLULAR MATRIX AND CELLULAR ADHESION TO 3D SCAFFOLDS

Extracellular matrix (ECM) proteins are substances that help create the micro niche in which cells reside and assist in their function. Examples of such proteins include laminin, fibronectin, vitronectin, collagen 1 and collagen IV. Most cellular tissue contains some form of ECM. Cells bind to extracellular matrix proteins via integrins on their cell surface. hESC, for example, express the integrins alpha3ß1 and alpha6, and bind preferentially to laminin (42). Laminin 1 is also the protein that acts as a morphogen for fetal pancreatic cells (43).

6.1. Islets

Islets contain basement membrane proteins including collagen IV and laminin. Integrin expression within the core of the islet is predominantly alpha6-beta1 heterodimer (44). This is a key laminin binding integrin complex indicating that beta cells require a basement membrane like matrix to support their function. Mouse islets seeded within PLGA scaffolds and transplanted into diabetic mice displayed significantly improved function when the scaffold was coated with collagen IV. Time to euglycemia and response to glucose challenge were both significantly faster for collagen IV coated scaffolds compared with scaffolds coated with fibronectin, laminin or non-coated control scaffolds treated with serum containing media (33). These results indicate that collagen IV is a superior ECM for enhancing glucose responsive insulin production. Scaffolds coated with either collagen IV, fibronectin or laminin maintained better islet structure and increased intraislet vascularisation when compare to non-ECM coated serum exposed scaffolds. Therefore coating of a scaffold with any of these ECM proteins improves islet acclimatisation post transplantation (33).

6.2. Stem and progenitor cells

The first step of culturing ESC on 3D scaffolds is to coat the scaffolds with appropriate ECM or matrices that allow cellular adhesion as well as to facilitate their differentiation towards the desired lineage. However, to date, much of the research that has been published relating to hESC differentiation has concentrated on the effects of soluble growth factors or genetic manipulation and only sparingly on the effects of adhesion to the ECM. Thus, there is a requirement for more research to be undertaken to investigate the role of adhesion and interactions of hESC with surfaces composed of ECM proteins in their pluripotency and directed differentiation.

Pluripotent hESC bind primarily to laminin (42, 45), but can also bind to fibronectin and Vitronectin (46). Laminin has been identified as the component of the ECM that is the most responsible for maintaining the pluripotency of hESC (45). It is also the most abundant component of matrigel (47), one of the most widely used matrices for ESC adhesion. Laminin has also been shown to be the matrix of choice when adhering pluripotent hESC onto PLGA scaffolds, in comparison to collagen I, collagen IV and fibronectin (42). Cells cultured on laminin coated PLGA scaffolds have the ability to differentiate towards definitive endoderm and undergo similar temporal genetic changes compared to those cultured on matrigel coated PLGA scaffolds and conventional monolayer cultures (48).

More interestingly, cells differentiated on 3D scaffolds expressed significant higher levels of definitive endoderm related genes *SOX17* and *FOXA2* compared to those differentiated on 2D monolayer cultures. This effect was observed regardless of whether hESC were attached to laminin or matrigel (48). This demonstrated the positive effect of the 3D culture system on the early stage pancreatic differentiation. We are unaware, however, of any published data analysing the efficiency of the 3D system on the later stages of pancreas development, namely the formation of PP from definitive endoderm (12). However, we are aware of the efficient differentiation of fetal mouse pancreas into insulin-producing cells once seeded onto PLGA scaffolds that are coated with both endothelial cells and fibroblasts (49). PLGA scaffolds coated with these cell types have been described previously as enhancing the formation of functioning cardiomyocytes from hESC (50).

Laminin has been the matrix of choice for pluripotent hESC adhesion; however, this matrix-preference appears to change as the cells develop towards specific lineages. The integrins alphaV and beta5 became highly expressed as pluripotent hESC differentiate towards definitive endoderm, underlining a potential role of integrins in ESC differentiation (51). Thus, it may not be appropriate to use a single matrix for ESC expansion and differentiation. Defined matrices should be used appropriately to cater the specific ECM requirements during each stage of ESC differentiation. The reason for using a defined matrix system rather than an undefined one like matrigel for ESC differentiation is that it is likely that only the former can support ESC differentiation towards the desired lineage. Such a system also potentially inhibits the formation of mixed populations of cells in culture, which can further improve the efficiency of differentiation.

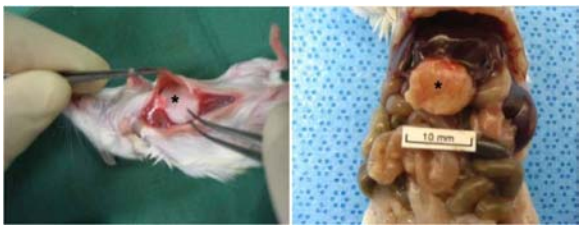

Figure 4. A PLGA scaffold (marked with an *) seeded with islets being transplanted into a BALB/c mouse (left) and at 41 days post transplant when the animal was euthanized (right). The scaffold post-transplant is well vascularised, with blood vessels visible on its surface.

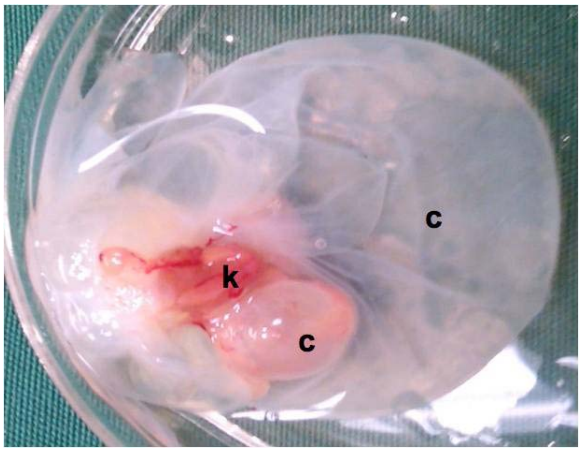

Figure 5. Teratoma at 12 weeks after transplantation of PP beneath the renal capsule of a NOD/SCID immunodeficient mouse. The surface of the grafted kidney can be vaguely seen (k); it is surrounded by loculated cysts (c).

7. SCAFFOLD VASCULARISATION

Islets contain a fine microvasculature obviously critical to their function in monitoring minute changes in blood glucose levels. Such cells seeded onto scaffolds and transplanted are vascularised by blood vessels of the host (Figure 4) (40). The promotion of both rapid vascularisation of islets and stem cells seeded on scaffolds with the host blood supply after transplantation is likely to be a key factor in the long term performance of the cells. A link between rapid vascularisation of implanted scaffolds and the embedding of growth factors within the biodegradable structure of the scaffolds has been demonstrated using vascular endothelial growth factor (VEGF). In a chicken embryo chorioallantoic membrane, angiogenesis assay scaffolds containing VEGF demonstrated a higher vascularisation index than untreated scaffolds (52). Scaffolds constructed from hyaluronic acid hydrogels are also able to support the function of a microvasculature both *in vitro* and *in vivo*, demonstrating anastomosis to the host circulation and blood flow through the hydrogel (53).

When hESC seeded onto scaffolds were transplanted between the liver lobules of immunodeficient mice, there was extensive vascularization both from host blood vessel incursion and differentiation of the hESC into endothelial progenitor cells (54). Confirmation of the human nature of these endothelial cells was achieved by showing staining with the human specific antibody CD34.

8. SCAFFOLD DEGRADATION

Following transplantation a scaffold will interact with the internal milieu of the recipient. This interaction will in most cases lead to the degradation and breakdown of the scaffold. Depending on what substance is used to manufacture the scaffold, this can have a significant effect on the performance of the graft and the integration of the transplanted cells into the host tissue environment. For example, scaffolds made from PLGA will degrade *in vivo* and release glycolic and lactic acid. Although lactic acid is a natural metabolic by-product, which is broken down in the citric acid cycle (38), the localised elevation of acidity may affect the surrounding milieu, potentially interfering with the function of surrogate beta cells. Moreover, the removal of fragments of the scaffold is by macrophages, which when attracted to the scaffold and become activated, produce pro-inflammatory cytokines, such as interleukin 1beta, tumour necrosis factor alpha and interferon , and chemokines, the macrophage inflammatory proteins. These agents have adverse effects on beta cells. Despite all of the above, there are some conditions which will support the long term function of islets seeded onto PLGA scaffolds, specifically adsorption of appropriate extracellular matrix proteins, such as collagen IV (33).

9. TERATOMA INCIDENCE

A defining feature of pluripotent stem cells, whether derived from embryos or de-differentiated from adult somatic cells, is their ability to differentiate into the three lineages ectoderm, endoderm and mesoderm. This occurs both in vitro and when the cells are transplanted. Large tumours that contain tissue from all three lineages are called teratomas and form when pluripotent stem cells are implanted, whether seeded onto scaffolds (54) or unattached to a device. An issue relevant to the transplantation of cells derived from pluripotent sources is the potential for teratoma formation (Figure 5), a consequence of remnant pluripotent cells remaining within the transplanted cell population. Two parameters appear to be indicative of teratoma formation, firstly the period of time/population doublings elapsing from when cells are removed from conditions known to promote a pluripotent phenotype and secondly the type of treatment cells undergo during the differentiation period (13). Currently, the most widely accepted and efficient protocol for derivation of PP suitable for transplantation requires approximately 2 weeks in vitro differentiation (11). However, a proportion of grafts (15%) taken from animals, which were transplanted with pancreatic endocrine progenitors derived using this two week differentiation protocol, displayed some form of teratoma like outgrowth (13). This is consistent with our own experience with a 19% incidence of teratoma formation after PP derived from hESC were grafted into immunodeficient mice.

Several methods can be incorporated to decrease the chance of teratoma formation including purification steps, extended *in vitro* differentiation or encapsulation. Our research has previously shown that encapsulation of pluripotent cells within barium alginate was sufficient to suppress teratoma formation (55). Prior to the acceptance of a therapy utilising PP for the treatment of T1DM in humans regulatory authorities will need to be as certain as possible that teratoma formation will not occur in the grafted cells and seeding cells within encapsulated scaffold devices is certainly a method for alleviating these concerns. It should be noted that the first human clinical trial using cells derived from hESC for the treatment of spinal cord injury involved injecting 4 patients with purified oligodendrocytes. Following these trials there have been no reports of teratoma formation in any of the treated patients (56).

10. IMMUNOPROTECTION

A key impediment to the function of transplanted tissue is its rejection by the host immune system. Successful therapeutic function of allograft islet transplants requires administration of immunosuppressive drugs to the recipients. These agents have significant negative side effects and may be detrimental to the function of transplanted tissue and the host (29). Transplantation of beta cell surrogates derived from hESC is likely to elicit a similar immune response to that observed with adult cadaveric islets, even though there is a disparity of the expression of histocompatibility antigens between the cell types. HLA class I antigens are minimally expressed and HLA class II not expressed on hESC (57), whereas both classes of antigens are on islets. However, when the hESC differentiate into PP and subsequently into mature beta cells, HLA expression would be expected.

A good method for avoiding immunorejection is to immunoisolate transplanted tissue within some form of prefabricated biocompatible device. These devices maintain export of therapeutic factors and waste products from the immunoisolated cells whilst selectively allowing the import of nutrients and oxygen but blocking the passage of immune cells and large immunoglobulins. Intravascular devices anastomose with the host vasculature and by doing so provide transplanted cells with a continuous supply of oxygenated blood. However, these devices are associated with significant risk of thrombosis and other complications and consequently there is now a preference to develop extravascular devices which do not directly anastomose with the host vasculature (58). Extravascular devices rely on sufficient positive flow of oxygen from the surrounding tissue to maintain viability of cells within the device. Therefore, devices must have favourable permeability allowing for trafficking of sufficient nutrients and oxygen from the surrounding interstitial tissue or adjacent vasculature to support the function of transplanted cells. Several type of macro-devices have been explored including planar membranes, hollow fibres and macro-capsules (58) but the field has predominantly concentrated on the development of microencapsulation of cells. Microencapsulation devices are generally manufactured from sodium alginate, with stabilization achieved by brief exposure to barium (59) or calcium. Some coat the surface of the alginate beads with cations such as polyornithine (60) to try and reduce the inflammatory response of the host to the capsules. A recent improvement has been reported with the use of a macro device that is transplanted subcutaneously. The device consists of a chamber for islets, which acts as an immunoprotective shield and an adjacent oxygen rich gas chamber. The oxygen in the gas chamber diffuses to the islet chamber at a controlled optimal rate ensuring that the transplanted islets receive sufficient oxygen to maintain function (61).

A method of incorporating scaffolds and microencapsulation devices is to seed the capsules rather than the cells onto the scaffolds. The scaffolds could be coated with anti-inflammatory agents to reduce any host response to the capsules (62) or with pro-angiogenic factors to enhance passage of nutrients to and waste products from the encapsulated cells. Alternatively, it is possible to place small scaffolds, seeded with cells, into microcapsules (63).

Immunoisolation devices are by design non-biodegradable and which directly contrasts with the characteristics of devices design to degrade over time and allow for long term incorporation of transplanted beta cell surrogates within the host tissue micro-environment. The need for immunoisolation may therefore compromise the design of carrier devices demanding precedence over less critical though desirable attributes.

When using stem cells as a tissue source, an alternative means of avoiding immune rejection is to utilise autologous cells. The use of expanded mesenchymal stem cells derived from the patient's own stem cell population, whether from peripheral blood or bone marrow, has been used to treat haematological disorders, such as leukaemia. However, no convincing source of adult stem cells, capable of expanding in great numbers and then differentiating into insulin producing cells, has yet been identified. The search is continuing and adipose tissue is being examined, with insulin-producing cells capable of being produced from this mesodermal tissue (64). Induced pluripotent stem cells (iPSC) are an alternative source of autologous cells.

Regardless of the cell source, any insulin-producing cells derived from these autologous cell sources will likely be killed by the autoimmune system, which previously destroyed the pancreatic beta cells of the recipient thereby causing T1DM. This process of reactivation of the autoimmune system after transplantation of tissues has been ably demonstrated when human islets are grafted (65). Encapsulation of the grafted cells theoretically should overcome this problem, since it is a cell mediated effect, and immune cells are too large to enter the microcapsules.

11. BIOREACTORS AND LARGE SCALE CELL PRODUCTION

One of the advantages of using ESC as a starting material for cellular replacement therapies is the unlimited

proliferative potential of these cells (22). It has been suggested that for a stem cell therapy to be clinically relevant the number of cells required per patient per dose can range from 10^4 to 10^{12} depends on the specific tissue/organ targeted, and in most cases, multiple doses are often required (66). Thus, to be able to expand the cells almost indefinitely is a major advantage.

In the context of using cellular replacement therapy as a treatment for type I diabetes, at least 9,000 islet equivalents per kilogram of body weight is often required (67). It has been reported by one study that each human islet consists an average of close to 1000 cells (68), and approximately 52% of which are beta cells (69). Based on this assumption, around 4.7 x 10^6 functional beta cell surrogates per kilogram of body weight will be required for each transplant. As such, for a 60-kilogram person, a total of 2.81 x10^8cells is needed. The typical culture density in a 2D environment is approximately 10^5 cells/cm^2 (66). This means that it would require up to 38 T-75 flasks to culture sufficient number of cells for the transplantation into one 60-kilogram person. In reality however, it was projected that a minimum of 10^8 cells per patient are required if transplanting ESC derived PP (70). Although this number is smaller than predicted above, significant scale-up from the conventional culture methods is still required.

The successful generation of iPSC means that each recipient could potentially be the donor of the somatic cells from whom the iPSC were derived (71). However, this also means that cells from multiple donors could not be pooled to reduce production costs. The resources required for producing the number of cells required for a clinical therapy, regardless of which type of pluripotent stem cell is used, becomes unmanageable if the cells were to be cultured in a conventional 2D environment. Thus, it is of upmost importance to develop alternative reproducible methods to scale up the differentiation and culture processes of ESC for clinical applications.

Various scaled-up culture systems have been developed, these include the use of multi-layered T-flasks (72) and automated microfluidic systems (73). However, both systems still utilize 2D adherent culture method, which is limited by surface area, and may not be sufficient to support the growth and differentiation of multiple batches of large number of cells in high consistency to meet the demands of current and future clinical requirements (66, 74). The use of bioreactors has been favoured as a potential method for scale-up stem cell cultures. Various bioreactors have been used in the past to successfully culture stem cells, including the culture of human bone marrow stem cells (75) and mammary epithelial stem cells (76) in perfusion bioreactors; the culture and differentiation of human (77, 78) and mouse (79, 80) ESC (mESC) in stirred-suspension bioreactors; and the culture and differentiation of adipose-derived stem cells in spinning flask bioreactors (81).

Bioreactors are devices that allow biological and biochemical processes develop under tightly monitored and controlled environments (82). A bioreactor aims to provide a consistent, dynamic environment with optimal level of mass transfer, oxygen tension and with minimal stress for culture of cells at high densities that is not achievable in a static environment. The capacity of a suspension bioreactor is not limited by surface area, rather, it is limited by volume; thus, a significantly larger number of cells can be cultured compares to previously described 2D adherent culture methods. It has been suggested that a conventional stirred suspension bioreactor can support the growth of 10^6 to 10^7 mammalian cells/mL (77), which means that a stirred suspension bioreactor with a capacity of just a few hundred mL is sufficient to house enough cells to meet the demand for a single beta cell transplant in a human.

The differentiation efficiency of mESC in suspension bioreactors can be negatively affected by fluid shear, which has been reported to maintain a population of cells in a transient pluripotent state (79, 80). This may have negative implications if the final cell product is to be used in a clinical setting. It has been suggested that cultured cells in capsules (83), micro-carriers or clusters can significantly reduce shear stress (84). hESC cultured on matrigel coated collagen micro-carriers have been shown to efficiently differentiate into definitive endoderm in a stirred suspension bioreactor (78). It has also been noted that hESC and mESC behave differently in dynamic culture conditions (84). The differentiation of hESC clusters into pancreatic endocrine cells was achieved efficiently in a rotational suspension bioreactor on a scale that is clinically relevant (85), no fluid shear related pluripotency was reported, demonstrating the enormous potential of using bioreactors for the production of pancreatic beta cell surrogates for human clinical applications.

The suspension culture of ESC in bioreactors normally requires the formation of aggregates (77-80, 85). As mentioned previously (48), the use of 3D biodegradable scaffolds has a positive effect of pancreatic differentiation. Suspension bioreactors (86) and perfusion bioreactors designed to accommodate 3D scaffolds have been developed and utilized to generate arteries (87), cartilage (88), osteoblasts (89) and myocardium (90).

Due to the dynamic nature of the culture environment in a bioreactor, cells are exposed to mechanical forces that are not present under conventional static culture conditions. Cell can be exposed to excessive shear forces in stirred suspension culture systems (86), and these forces can cause cell death. In contrast, low shear stress can result in the excessive formation of aggregates (91-94). This will subsequently render the oxygen tension and mass transfer efficiency of the culture system sub-optimal, and consequently negatively affect the proliferation and differentiation of the cells (82, 95). The culture of cells seeded on 3D scaffolds in a perfusion bioreactor can suffer from cell “wash out” (86), where cells directly exposed to the flow can be removed by the fluidic force. This results in a non-uniform reduction of cell density, which can significantly affect the progression of tissue formation as well as its function (86), and the consistency of the tissue formed (86). Therefore, considerable effort is required to determine the optimal

conditions suitable for the generation of beta cell surrogates from hESC in bioreactors.

The successful generation of functional beta cell surrogates *in vitro* from hESC for clinical use is a multifactorial process. It requires the combination of appropriate ECM for cellular adhesion and differentiation, correct growth factors for timely inductions, defined media to support cell growths at various stages of development, scalable culture system to produce a large number of cells with batch to batch consistency, and the implementation of GMP practices at all steps. As more and more research has been conducted for the purpose of pushing stem cell based therapies towards the clinic, pancreatic beta cell replacement therapies using hESC as a source material will no doubt benefit from the technological advances made in the fields of bioengineering and biomaterials for the development of a suitable transplant vehicle.

12. CONCLUSIONS

This review summarizes the state of the art regarding the seeding of islets, stem cells and pancreatic progenitors on 3D scaffolds. It shows there is a benefit in doing so, with more efficient production of definitive endoderm and pancreatic progenitors from human pluripotent stem cells. It also demonstrates that blood glucose levels can be more efficiently normalized in diabetic mice if mouse islets are seeded onto scaffolds before being transplanted. Such scaffolds may be of use in bioreactors that will be needed to produce the large number of pancreatic progenitors needed for human diabetic trials. They may also be implanted into diabetic people, but before doing so, a means of preventing rejection of the cells will be needed, perhaps by placing the cells in microcapsules made of alginate.

13. ACKNOWLEDGEMENTS

We wish to thank A/Professor Hala Zreiqat, Dr Gayathri Sundaram and Dr Jayne Foster for assistance with Figures 2, 3 and 4 respectively. We are grateful for financial assistance from the following organizations which assisted the experiments we carried out: Australian Foundation for Diabetes Research, Juvenile Diabetes Research Foundation, National Health & Medical Research Council of Australia, Rebecca L. Cooper Medical Research Foundation, and Sydney Medical Research Foundation.

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Key Words: Diabetes, Stem cells, Tissue Engineering, Biodegradable Scaffolds, Differentiation, Review

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