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Generation of Insulin Producing Cells for the Treatment of Diabetes

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1. Introduction

The are approx. 177 million people suffering from diabetes worldwide and this number will be doubled by 2030. Patients with type 1 diabetes require multiple injections of insulin daily with doses careful adjusted on carbohydrate intake, level of activity and stress. Often there is a mismatch between insulin requirements and the calculated dose, resulting in hyper or hypoglycaemia. Between 20-30% of patients however suffer from recurrent hypoglycaemia, which may require third party help (Geddes et al. 2008). Islet transplantation has been a promising therapy since the "Edmonton protocol" was published in 2000 (Shapiro et al. 2000). However, numbers of transplants remain low for a variety of reasons (Shapiro et al. 2006). One major hurdle to more widespread provision of this treatment remains a shortage of supply of donor organs, with only 25-30% of isolations resulting in islets of sufficient quantity and quality to be used in clinical transplantation, indicating a huge waste of valuable resources (Nano et al. 2005). Studies using animal models have found the evidences of neogenisis of beta cells under non-physiological condition as well the limited proliferation capacity of beta cells. Unfortunately, human beings cannot be manipulated to generate insulin-producing cells. There are increasing evidence suggested that glucose response insulin-producing cells could be generated in large quantity for human use. The following are a few possible aspects that can be explored for this purpose.

1.1 Beta cell replication and regeneration

There are substantial evidences suggesting that the adult pancreas can generate new β-cells in response to pancreatic damage or increased demand for insulin (Wang et al., 1995; Bonner-Weir et al., 1993; Guz et al., 2001). The source of new β-cells is thought to be the replication of existing β-cells under normal growth condition (Dor et al., 2004). However, the capacity of mature β-cells to proliferation and then re-differentiate back into β-cells has not been demonstrated *in vitro* and it still remains a challenge. In addition to the replication, there are mounting evidences indicated that regeneration of beta cells also contribute significantly to the new beta cells when pancreas is under non-physiological conditions such as partial pancreas duct ligation (Wang et al., 1995) or partial pancreatectomy (Finegood et al., 1999). However, such mechanisms are believed to have little implication to diabetes patients, as it is not possible to mimic the situations in animals to humans. Furthermore,

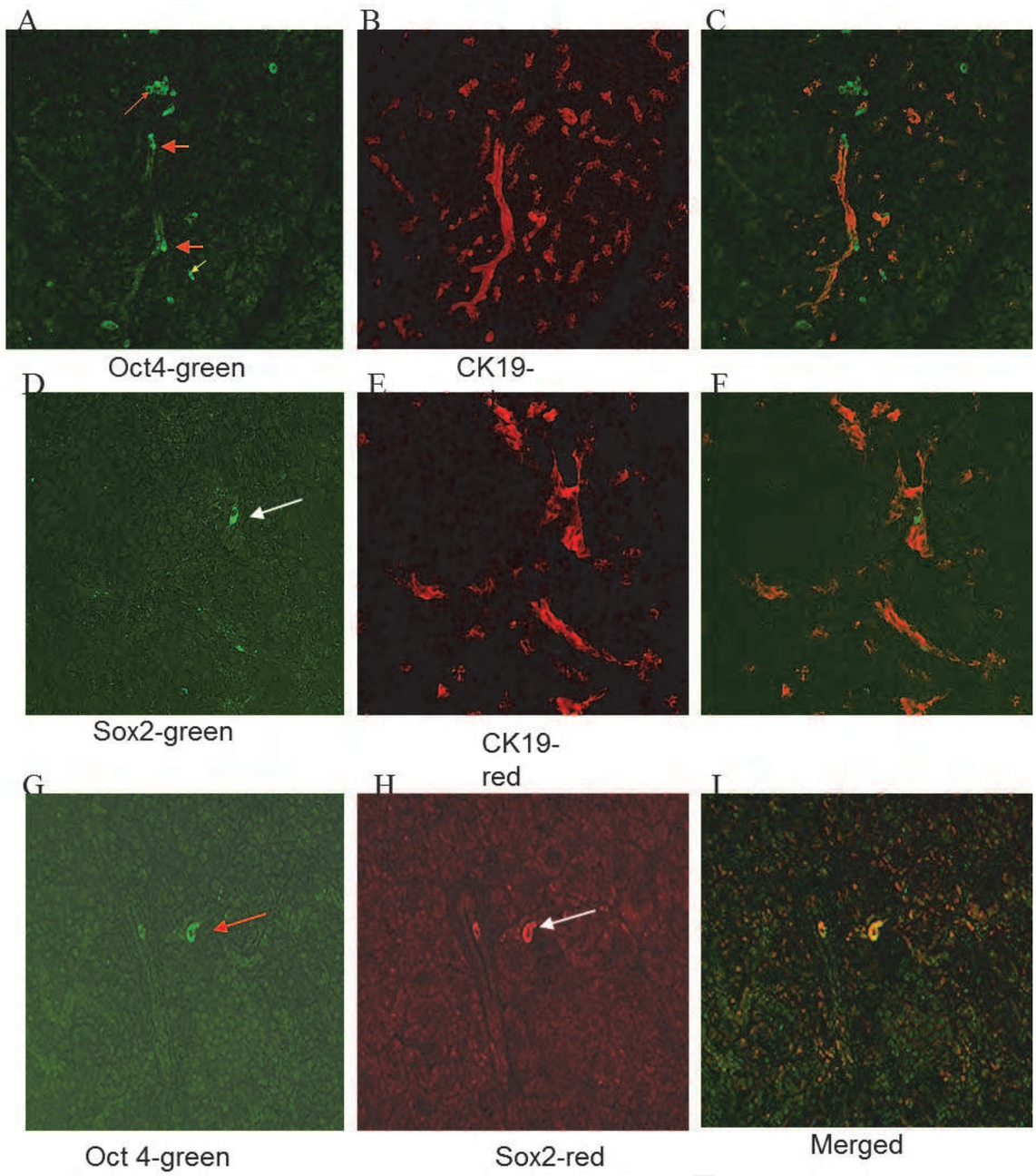
even though there is regeneration of beta cells; the patients need immunosuppressive drugs to control the self-destruction of beta cells by autoimmune mechanisms. In addition to the beta cell proliferation, whether there are other cell sources for the beta cell neogenesis is not clear. It has been shown that human adult pancreases contain cells expressing Oct4 and Sox2 proteins (Zhao et al., 2007). The expression of Oct4 and Sox2 is thought to be related to the stemness of stem cells in human pancreas. The location of these cells in the small ducts is interesting (Fig 1) as it reflects the development of pancreas. Whether these cells are a part of the undifferentiated stem cells retained in ducts during the formation of pancreas and are the sources of new beta cells in adulthood remains unknown to us. The question is whether these cells are truly functional adult stem cells? If they are, how these cells contribute to the balance of beta cell number warrants further investigations. Insulin positive cells were indeed observed in the pancreas ducts (Bonner-Weir et al., 1993; Dudek et al., 1991; Pour 1994; Bouwens and Pipeleers 1998; li et al., 2010) and the findings that cells derived from rodent and human islets were multiple potency (Smukler et al., 2011) have further confirmed the possibility of presence of stem cells in adult pancreas. For this reason, human pancreatic ducts have been the target material for insulin-producing cells (Gmyr et al., 2004). Unfortunately the experiment protocol seemed not be optimal enough and generating insulin-producing cells from these duct cells is remaining illusive. Further investigation is required to determine whether there would be possible means to increase beta cells from the stem cells to treat diabetes in newly diagnosed diabetes patients together with immune modulation drugs.

1.2 Immunosuppression approach

When the patient is newly diaganisd as type 1 diabetes, there is often associated with a "honey-noon" phenominon. It is thought that there are new beta cells generated during this transient phase of disease. Earlier clinical trial using immunosuppressive drugs to inhibit the T cells did show the improvement in insulin secretion but at the price of worsening renal functions (Mirouze et al., 1986; Mandrup-Poulsen et al., 1990). These are believed to be the side effects of the immunosuppressive drugs to the kidneys while modulating the autoimmunity to protect beta cells. In 1990s, the most widely used drugs is cyclosporine, which is known to be toxic to beta cells (Hahn et al., 1986), will also damage the beta cells overtime. The trials with less cell toxic drugs such as anti-CD3 or anti-CD4 antibodies also showed some benefits in altering the disease course (Chatenoud et al., 1993; Phillips et al., 2000; Herold et al., 2002) but the hope of increasing new beta cells suficient to cure the disease still meets difficulty (Keymeulen et al., 2005). It is believed that the time to initiate such treatment is crucial as the ability to increase new beta cells is limited. This treatment could be used to prevent type 1 diabetes in risk population when there is still a surficient number of beta cells and with potential renewing beta cells, if such a population is identified.

1.3 Pancreas organ transplantation vs. islet cell transplantation

Human pancreas transplantation has been the main option for replacing the lost beta cells in type 1 diabetes patients since 1960s (Kelly et al., 1967; Hermon-Taylor 1970). Even now it is still the best option to treat diabetes. The insulin independency following pancreas organ transplantation is around 70-90% when the surgical procedures are successful. However, it is a major surgical procedure and is normally reserved for kidney and pancreas



Typical immunohistochemical staining images of human pancreas sections for the Oct4, Sox2 expressing cells and their localization relevant to CK19^{+ve} cells. Panel A and G show Oct4^{+ve} cells (green) within the ductal structures and their surrounding area in a scattering pattern. Majority Oct4^{+ve} cells were shown cytopalsmic staining with only small number cells shown nuclei staining (arrowed). B and E show the CK19^{+ve} duct cells (red) and C is the merged of A and B. Panel D (green) and H (red) show Sox2 positive cells. F is the merge of D and E. Panel I is the merged of G (Oct4^{+ve}, Green) and H (Sox2^{+ve} Cells, Red), showing the colocalization of Oct4 and Sox2 positive cells.

Fig. 1. Immunohistochemical statining of the Oct-4 and Sox2 cells in human adult pancreas sections

simultaneous transplantations just because it is a big operation procedure. Most old patients are not suitable for this approach as they are unable to tolerate the surgical procedures. The operation itself is associated with 10% mortality and mobility; often the patients need a second or third operation to correct the problem of exocrine enzyme leakage.

Human islet transplantation provides an alternative mean to restore the lost beta cells and it become a realistic option for a group of type 1 diabetes patients with bristle diabetes condition, especially for the group of patients with hypoglycaemia unawareness (Shapiro et al. 2000). The advantage for this approach is that it is a minor and less invasive procedure and a more safe approach for the replacement of lost beta cells. The disadvantages are that human pancreas is to be digested with a blend of collagenases and specialized proteases and human islets are purified from the exocrine tissues (Ricordi et al., 1989). Some islets are damaged during the isolation procedures. In addition, approximately 20-50% islets are lost under current density centrifugation purification techniques, which is based on the density differential between endocrine cells and exocrine cells. Therefore, it needs 2-3 pancreases to provide enough islet cell mass (~10,000 Islet IEQ/kg body weight) for one recipient. The human islets are then transplanted into the patient's liver via port vein, where the islets will set down in liver. The islets will function in the higher insulin concentration environment as comparison with those within pancreas. Therefore, this will require islets to work harder and lead to exhaustion of islets themselves. As the islets are digested away from the exocrine tissues, they are also cut away from the vascular system, which provides islets with essential nutrients, oxygen and neuron-regulating molecules and need to be revisualization following transplant. During the period of avascular state, the islets are very vulnerable to any attack, such as inflammatory factors and the cytotoxicity of the immunosuppressive drugs, as the cells take in nutrients and oxygen through passive perfusion, which is very inefficient. Since the body immune system destroys tissues it recognises as "foreign" and the nature of autoimmune disease in the patients, immunosuppressive drugs are given to the patients before, during and after transplant to protect the grafts from allo-rejection and the reoccurrence of autoimmunity. The immune regulation medication has several drawbacks currently. First, the immunosuppressive drugs have significant side effects and some patients cannot tolerate one or another immunosuppressive regimens. The patients have to take the drugs life long in order to protect the islet grafts and to prevent from getting sensitisation against donor tissues. There would be some implications for the risk of tumour generation, as the recipients may not have the full immune capacity to fight tumour cells although there is no such report yet. For the reason of toxicity of the immunosuppressive drugs, this approach is only suitable for a small group of patients. It is not yet suitable for young children simply it is not worthy taking immunosuppressive drugs. Secondly, the drugs also show some degrees of toxicity to beta cells and the drugs will reduce the islet cell mass overtime. The long-term prospect of islet transplantation currently is not yet satisfactory, with only ~13% of transplant patients remaining insulin independence for >5 years, although most patients still have endogenous C-peptide product and benefit from the better control of their blood glucose (Ryan et al., 2005). Thirdly, as to the drawback mentioned above, it requires 2-3 donor organs to generate enough islet cell mass to reach to >10,000 islet IEQ/kg body weight of the recipient. The large islet IEQ is needed because some islets will not survive during the avascular state, which deprive themselves of essential nutrients and oxygen, and the toxicity of immune modulation agents. This will obviously worsen the situation on the already limited supply of donor organs. Fourthly, the reoccurrence autoimmune attack on the grafts developed overtime, which seemed to be able

to escape the current immunosuppressive regimens applied. The future work should concentrate on protecting islets during and post-transplantation and development of less toxic immune suppression drugs.

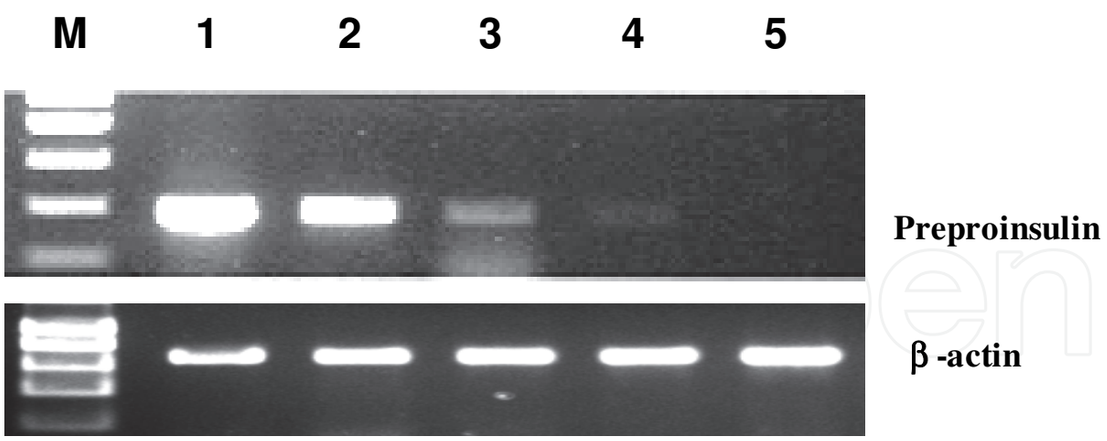
Despite the drawback mentioned above, for patients with bristle diabetic conditions and patients with pancreatectomy (due to prolonged pancreatitis), the beta cell replacement still remains the best option. To make this beta cell replacement as a wider applicable approach for the treatment of diabetes patients, the limitation of glucose responsive insulin-producing cells is the key obstacle and must be addressed. For patients with type 2 diabetes, current islet cell transplantation is insufficient as the demand for insulin-producing cells for these patients is far greater than type 1 diabetes patients. However, this approach could be applied to patients with type 2 diabetes, if the cell source of insulin surrogates is unlimited. For the later, there is only one need, that is, to prevent the allo-graft rejection.

2. Generation of glucose responsive insulin-producing cells

Many type of cells have been used to generate glucose responsive insulin-producing cells with limited success. These include the differentiation of embryonic and adult stem cells and transdifferentiation from other types of cells into insulin producing cells (Fujikawa et al., 2005; Zhao et al., 2005 and 2008; Kroon et al., 2008; Boyd et al., 2009; Tateishi et al., 2008; Zhou et al., 2008; Gabr et al., 2008; Zhang et al., 2009; Cai et al., 2010). However, the success has been limited to small animal models. The focus of this chapter is to briefly discuss the potentiality involved in our laboratories.

2.1 Islet cells replication *in vitro*

Islets of Langerhans are the endocrine mini-organs consisted of 4 major different endocrine cells: α-cells controlling the release of glucagon; β-cells for insulin, δ-cells for somatostatin and the pp cells-polypeptide. These 4 types of endocrine cells work together in a complex interplay in the maintaining of homeostasis of blood glucose level. Islet cells need to stay together as a cluster to function better as indicated by the finding that single Min 6 (mouse beta cell line) cells do not express and secret insulin as well as those when the Min 6 cells are forming as a cluster-called pseudoislets (Hauge-Evans et al., 1999). Human islets cultured in monolayer tend to lose the capacity gradually to express insulin within 5 weeks (Fig.2). Human islet cells cultured in 3-dimension seemed to be able to retain beta cell phenotype. However cells, particularly those at the centre of the clusters, will die due to necrosis if the cells cultured as 3-dimensional manner for too long. This is because that cells located in the centre of the 3-dimension will depend on the perfusion of nutrients and oxygen, which is very insufficient. We therefore developed a method to culture the cells in a rotation manner between monolayer culture (2-dimension) and cluster culture (3-dimension), the cells have acquired the survive signal through the 2-dimensional culture and can also maintain the capacity to express insulin through the 3-dimensional culture. The cells can maintain this capacity to express insulin and respond to glucose challenge for >4 months *in vitro*. When the cells were transplanted into SCID (an immune deficient mouse) mice, rendered diabetes by the injection of straptozotosin (STZ), they were able to correct the hyperglycaemia and maintaining the homeostasis of blood glucose (Fig. 3; Zhao et al., 2002). The beauty of this model is to provide a prototype model to analyse beta cell differentiation. The ability of beta cells to proliferate *in vitro* has been demonstrated in many studies (Beattie et al., 1999; 2000



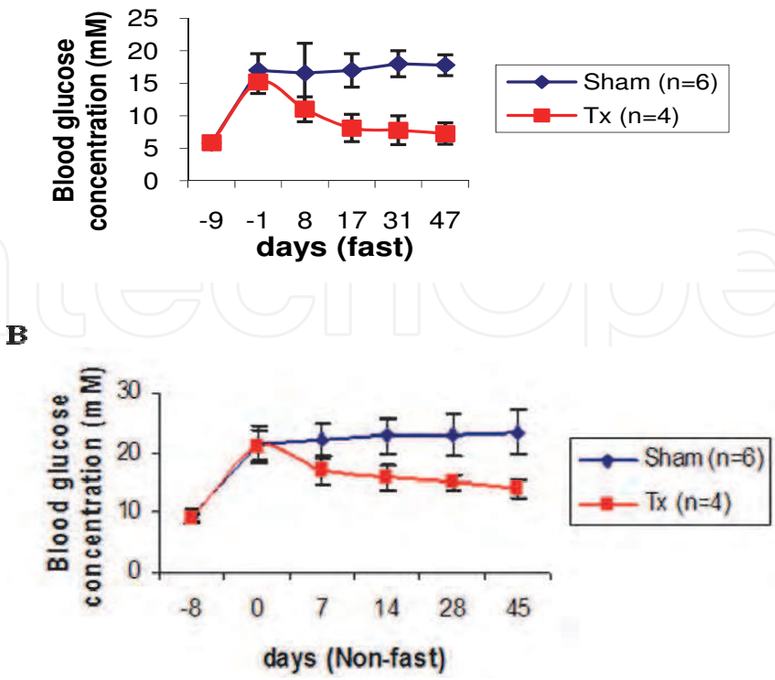
Semi-quantitative RT- PCR analysis. 5 weeks continuously monolayer cultured human islet cells were induced to express insulin. Total RNA were isolated from each different condition. cDNA derived from 4ng of total RNA were used to amplify preproinsulin and actin. 5 = 5weeks monolayer cultured human islet cells.

Fig. 2. Analyses the expression of insulin in continual monolayer culture

and 2002; Halvorsen et al., 2000; Gershengorn et al., 2004; Lechner et al., 2005; Ouziel-Yahalom et al., 2006;) and was directly confirmed in a study with GFP labelled beta cells using a lentinvirus system (Russ et al., 2008). However, the purpose of *ex vivo* expansion of human islets is to increase the glucose responsive insulin-producing cells for research and eventually for transplantation to treat diabetes. Unfortunately, making the proliferated insulin-producing cells to express insulin again in a glucose responsive manner is still a big challenge to us. Using a lentinvirus labelling system developed by Russ and colleagues, we were able to show that a small percentage of beta cells (GFP expressing cells) were found to be positive for insulin and Ki67--a proliferation marker *in vitro* (Fig. 4; Zhao et al., unpublished). This result illustrated that beta cells have the potential to be expanded *ex vivo*. The question is how to make this more efficient to achieve large number of cells for the treat of diabetes patients.

2.2 Human exocrine cells

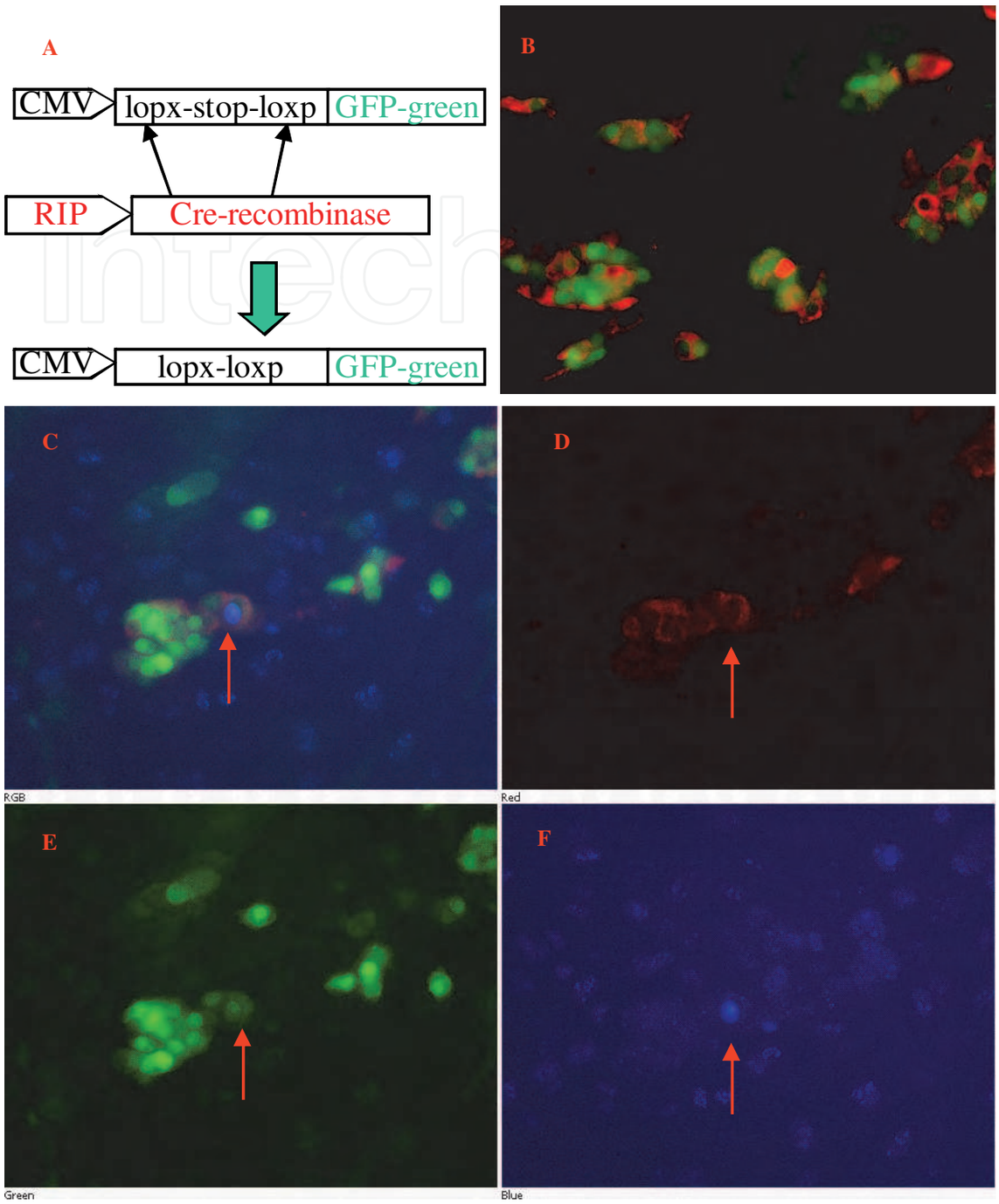
From the development point of view, exocrine cells are the excellent candidate materials for insulin-producing cells as they share the same origin with the pancreatic endocrine cells (Slack 1995). Mouse exocrine cells have shown the flexibility to be converted into insulin-producing cells following chemical treatment (Lardon at al., 2004; Baeyens et al., 2005; Zhou et al., 2008). Therefore, there is good reason to believe that it is possible to convert human exocrine cells into endocrine cells. In terms of cell number, exocrine cells consist of 90% pancreatic cells with approximate 40-50 folds of their endocrine cell count apart, which consists of less than ~2% of total pancreatic cells. Secondly, it is already available. Following human islet isolation, the exocrine cells will be the waste material and are discarded at end. Thirdly, following dedifferentiation there is no obvious difference between the two types of cells. Both cells began to express CK19—a ductal cell marker. This is interesting because that both types of cells are derived from ductal cells during pancreas development (Slack 1995). Whether to differentiate into exocrine cells or into endocrine cells is under the complex interplays of complex genes at that stage. Gene Ptf1a may play a big role in the



Blood glucose level (mM) of the STZ induced diabetic SCID mice before and after transplantation of long term cultured islets or sham control. Time of transplantation and sham was assigned as 0 day (d) and the time before surgery was indicated as –d. A shows the overnight fasting blood glucose level, solid square = islet cell transplanted (Tx), and the solid diamond shape = sham control. B shows the non-fasting blood glucose levels, solid square = islet cell transplanted (Tx), and solid diamond shape = sham control. The decrease in blood glucose levels was significant in both fasting and non-fasting with student t pair-test at $P<0.023$ and $P<0.002$ respectively. The difference in blood glucose levels between transplantation and sham groups was also significant ($P<0.02$and 0.023 respectively).

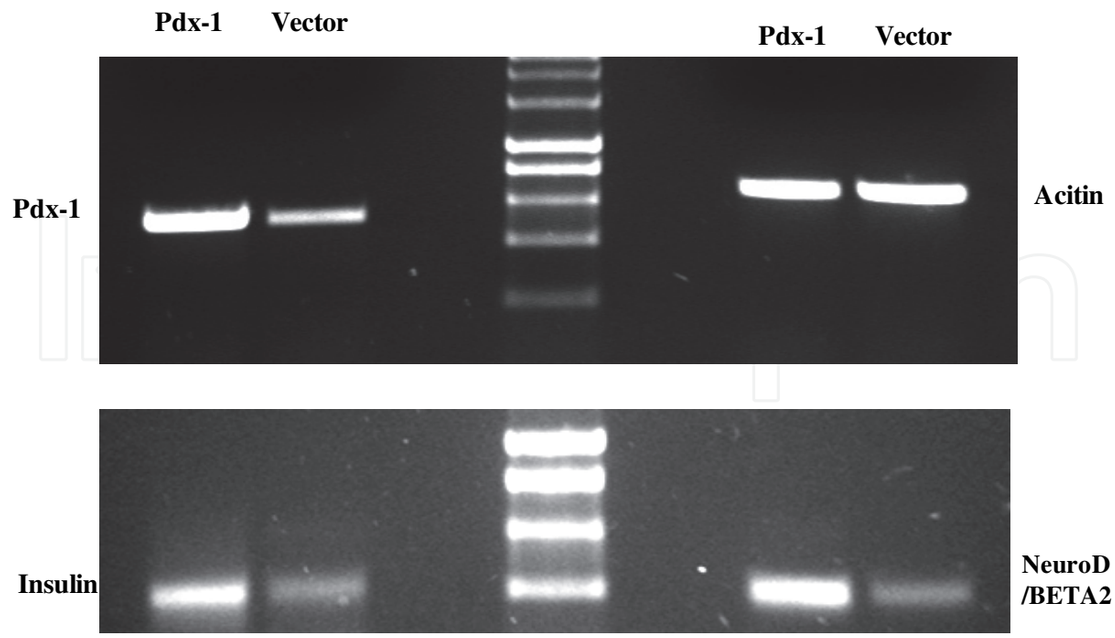
Fig. 3. Analyses of the blood glucose levels in mice transplanted with long term cultured human islet cells

determination of cells to exocrine cell fate (Dong et al., 2008). The mechanism of how exocrine cells being converted into endocrine cells is not clear and under our experimental conditions, it requires the gene products from beta cells and the right differentiation environments. Under the Pdx-1 gene influence, exocrine cells express some beta cell phenotype marker following differentiation induction with a cocktail of differentiation inducers (GLP-1, activin A, betacellulin, nicotinamide and glucose, Zhao et al., 2005) and expressed low level of insulin (Fig. 5) *in vitro*. The time to introduce Pdx-1 is seemed to be critical, although we did not have biomarkers to match this window exactly for the gene induction. We speculated that a short window existed during the dedifferentiation of exocrine cells. During which, exocrine cells would be more ready to be induced to express beta cell markers. The cells showed a glucose response *in vitro* when challenged with glucose. But the response was not typical as the background secretion is too high and the challenge secretion is too low in comparison with islet cells, suggesting the insulin-producing cells were immature. As predicted, these cells matured further following transplant in SCID mice, rendered diabetes with the injection of STZ solution. This approach is sufficient to work in small laboratory animals with ~40% mice recovered from the mild



Genomic labelling of human beta cells and differentiation induction assessment. Panel A: the methed used in the labelling of human beta cells (Russ et al., 2008). Panel B: Control experiment of differentiation induction in the labelling beta cells. Beta cells were labelled as green and the cells were staining for insulin (Red). Panel D, E, F were images catched for red (insulin, D), green (beta cells, E) and blue (for Ki67 protein F). Panel C was the merged images of D, E, F. The proliferated beta cell was indicated by the arrow.

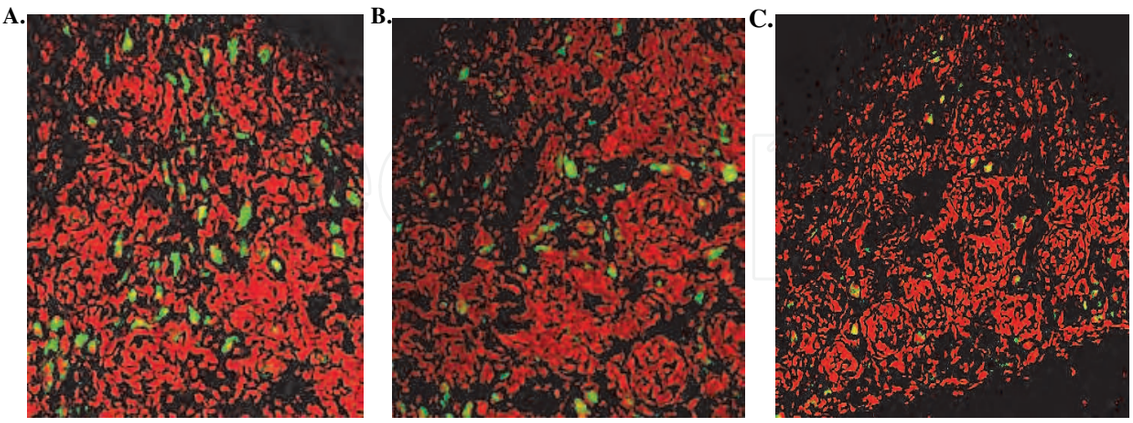
Fig. 4. Analyses the potential of proliferated adult human beta cells to reexpress insulin



The dedifferentiated human pancreatic exocrine cells expressed insulin and NeuroD1/Beta 2 genes after transfection with Pdx-1 gene and differentiation induction, analysed by semi-quantitative RT-PCR.

Fig. 5. Expression of beta cell phenotype genes in manipulated human non-endocrine pancreatic cells in vitro

diabetes. The grafts showed that glucagon and somatostatin were also expressed in addition to the insulin (Fig.6), indicating that exocrine cells can be converted into whole pancreatic endocrine cells, not just insulin producing cells. Further works are required to explore the full potential of exocrine cells in order to create enough insulin producing materials for the treatment of diabetes.

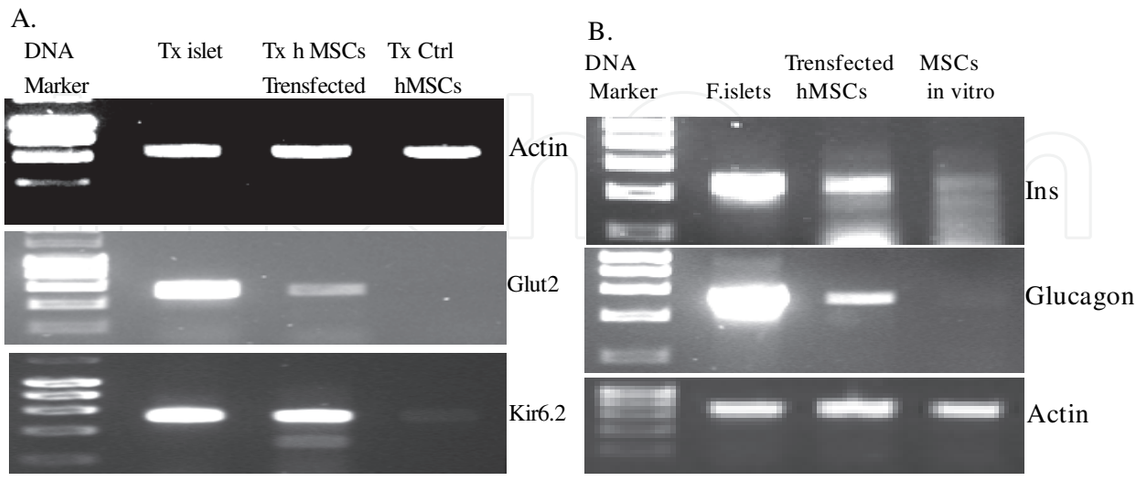


Immunohistochemical staining of the grafts (visulised by confocal microscope) for markers of the pancreatic endocrine cells. Insulin (A, 15±6.7%), Glucagon (B, 8±2.3%) and Somatostatin (C, 3±1.85%) positive cells are shown in green (FITC) and nuclei are stained with propidium iodide (red). Original amplification x40

Fig. 6. The expression of pancreatic endocrine cell hormones in the transdifferentiated cells.

2.3 Mesenchymal stem cell

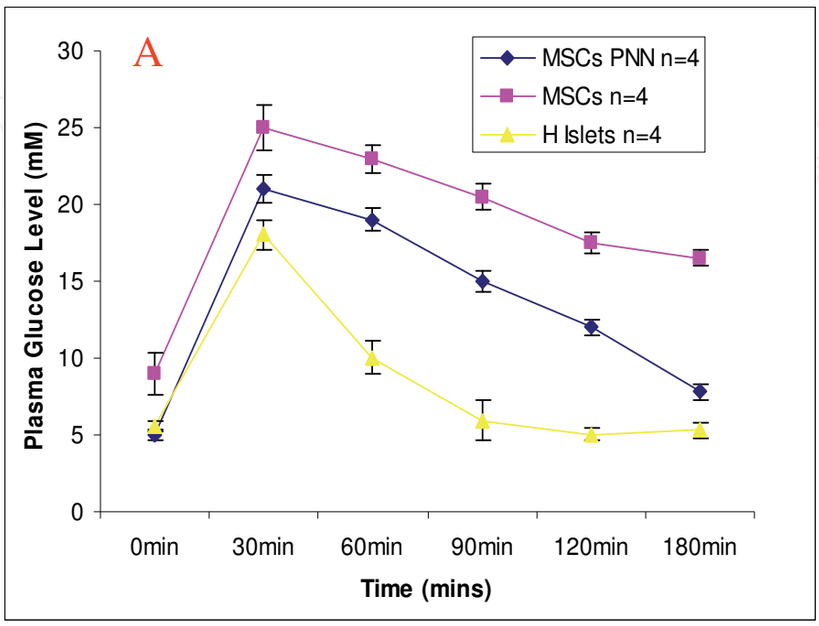
Mesenchymal stem cells (MSCs) are the nonhematopoietic multipotent progenitor cells found in various adult tissues. Bone marrow derived MSC cells are those cells can adhere to plastic culture flask and proliferate *in vitro* (Kadiyala et al., 1997). The cells have the potential to be expanded *in vitro* in large quantity. MSC under specific differentiation environments can differentiate into cells with phenotypes of many specific tissues, such as bone, adipose and neurons (Pittenger et al., 1999; Deans and Moseley 2000; Deng et al., 2006; Gabr et al., 2008). MSC has the capacity to modulate host immune cells (see review papers in Bunnell et al., 2011) and express molecule help themselves evade the immune attack (El Haddad et al., 2011). The ability of MSC to become insulin-producing cells has been in debates and been controversy (Lechner et al., 2004; Taneera et al., 2006; Butle et al., 2007; Moriscot et al., 2005; Timper et al., 2006; Lee et al., 2006; Karnieli et al., 2007; Lavazais et al., 2007; Hasegawa et al., 2007; Denner et al., 2007). Human bone marrow MSC express low level of Oct4 and Sox2 as well Pdx-1—an important gene involve in the development of beta cells and the maintaining of beta cell phenotype in adult, indicative that these cells could be differentiated into glucose responsive insulin- producing cells. Under the influence of the gene products of Pdx-1, NeuroD1/BETA2 and Ngn3, the MSC express insulin gene *in vitro* following differentiation induction with a cocktail of differentiation inducers (GLP-1, activin A, betacellulin, nicotinamide and glucose). The cells seemed lacking insulin storage capacity and secreted insulin as it was synthesised. Therefore these cells were not glucose responsive *in vitro*. Following transplantation into SCID diabetes mice, the cells were able to mature further and expressed most beta cell phenotype gene markers, such as Glut-2 and Kir6.2 (Fig. 7A) and insulin processing enzymes, but the cells did not express Sur1 gene and therefore did not have a functional K-ATP channel. Interestingly the cells also expressed glucagon gene, indicating that some cells also differentiated into α-cells (Fig.7B) in addition to insulin producing cells. Nevertheless, the hyperglycaemia in the transplanted mice was corrected despite that the transplanted mice had some impairment in glucose tolerant in comparison with mice transplanted with human islets (Fig. 8 and Zhao et al., 2008). These

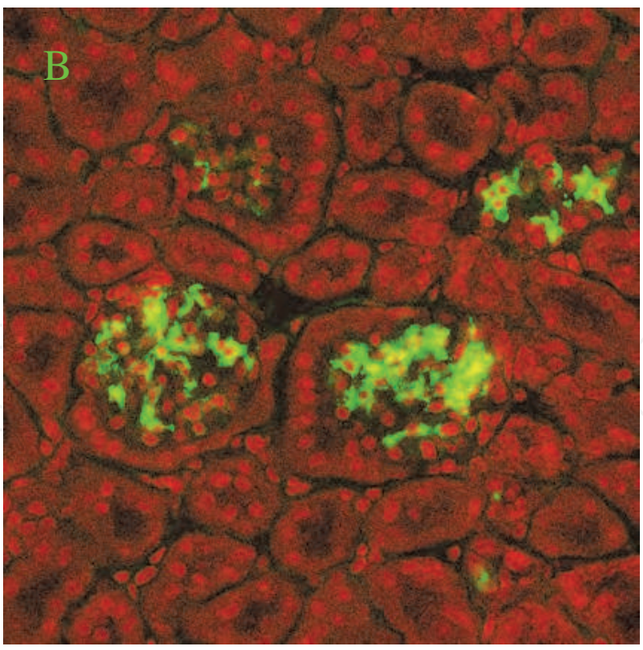


Analyses of the expression of β and α cell genes by semi-quatatative RT-PCR on kidneys transplanted with the cells.

Fig. 7. Gene expression analyses on the transplanted kidneys by semi-quatative RT-PCR

data suggested that the MSC achieve partial glucose responsiveness through non-ATP K channel mechanism. The MSC did not form any teratoma structures *in vivo*; making it an idea candidate to be beta cell surrogates for the treatment of diabetes.





Panel A. Mice transplanted wtih the Pdx-1, Ngn3 and euroD1/Beta2 genes shows response to glucose challenge During a glucose tolerlant test. Panel B shows the manipulated human MSC expressing insulin (green fluorescence) in diabetic mice.

Fig. 8. Functional analyses in mice transplanted with human MSC cells

3. Conclusion and future direction of investigation

There are mounting evidences including our own data described above suggested that insulin-producing cells can be generated from many cells through the differentiation of stem cells (embryonic stem cells to adult stem cells) or via the transdifferentiation mechanisms. However, there are challenges ahead. The most urgent tasks are 1) to optimise the differentiation protocols to make them more efficiency by optimising the differentiation inducers and the extracellular environments; 2) to identify the molecular mechanisms associated with the differentiation to allow the translation of bench discovery to bedside treatments; 3) to enhance the efficacy to increase the cell mass for human usage.

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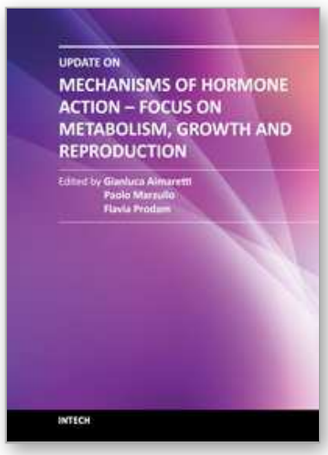

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The purpose of the present volume is to focus on more recent aspects of the complex regulation of hormonal action, in particular in 3 different hot fields: metabolism, growth and reproduction. Modern approaches to the physiology and pathology of endocrine glands are based on cellular and molecular investigation of genes, peptide, hormones, protein cascade at different levels. In all of the chapters in the book all, or at least some, of these aspects are described in order to increase the endocrine knowledge.

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