Review Article

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Stem cells to replace or regenerate the diabetic pancreas: Huge potential & existing hurdles

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Various stem cell sources are being explored to treat diabetes since the proof-of-concept for cell therapy was laid down by transplanting cadaveric islets as a part of Edmonton protocol in 2000. Human embryonic stem (hES) cells derived pancreatic progenitors have got US-FDA approval to be used in clinical trials to treat type 1 diabetes mellitus (T1DM). However, these progenitors more closely resemble their foetal counterparts and thus whether they will provide long-term regeneration of adult human pancreas remains to be demonstrated. In addition to lifestyle changes and administration of insulin sensitizers, regeneration of islets from endogenous pancreatic stem cells may benefit T2DM patients. The true identity of pancreatic stem cells, whether these exist or not, whether regeneration involves reduplication of existing islets or ductal epithelial cells transdifferentiate, remains a highly controversial area. We have recently demonstrated that a novel population of very small embryonic-like stem cells (VSELs) is involved during regeneration of adult mouse pancreas after partial-pancreatectomy. VSELs (pluripotent stem cells in adult organs) should be appreciated as an alternative for regenerative medicine as these are autologous (thus immune rejection issues do not exist) with no associated risk of teratoma formation. T2DM is a result of VSELs dysfunction with age and uncontrolled proliferation of VSELs possibly results in pancreatic cancer. Extensive brainstorming and financial support are required to exploit the potential of endogenous VSELs to regenerate the pancreas in a patient with diabetes.

Key words Diabetes - ES cells - islets - pancreas - stem cells - VSELs

Diabetes is one of the major non-communicable diseases in the world with majority of patients belonging to India, China and USA. Along with associated complications like heart disease and stroke, diabetes results in increased morbidity and mortality and it is expected that by the year 2025, India alone will have more than 70 million diabetics[1,2]. Diabetes is a metabolic disorder associated with progressive loss or dysfunction of β-cells of pancreas. Onset of type 1 diabetes mellitus (T1DM) occurs when the β-cell mass is reduced to less than 20 per cent due to autoimmune effect, whereas the declining β-cell mass is unable to meet the age-related increased insulin demands of the body in type 2 (T2DM) as a result of insulin resistance and in due course the β-cells are lost by apoptosis. Thus, in both T1 and T2DM, restoration of a functional β-cell mass constitutes the central goal of diabetes therapy. Besides a change in lifestyle and administration of insulin sensitizers to T2DM patients, an unmet need exists to regenerate the pancreas (implying regenerating

healthy β-islets). In case of T1DM, transplantation of islets (derived from cadavers, foetal or from non-human xeno-source or from the stem cells) may help whereas in case of T2DM attempts are being made to facilitate endogenous regeneration of islets in the pancreas. There was a rush in the clinics to transplant autologous bone marrow stem cells and mesenchymal stem cells (MSCs) and handful of pilot studies showed marginal benefit. Basic scientists are still divided as to whether pancreatic regeneration occurs by reduplication of existing islets, trans-differentiation of ductal epithelial cells or whether neogenesis of islets involves stem cells. Elaborate studies conducted over more than a decade, from Harvard University[3] have demonstrated a role for PDX-1 positive progenitors during regeneration of mouse pancreas and lineage tracing studies have shown that these cells possibly arise from the ductal epithelium by de-differentiation involving regression of cells to an earlier stage of a progenitor which can differentiate into islets and acinar cells during regeneration[3]. A study reported in 2013[4] using tamoxifen independent INS^{Cre}mTmG compound mice could not generate any evidence by flow cytometry in support of neogenesis of β-cells. We have recently demonstrated how very small embryonic-like stem cells (VSELs) are involved during pancreas regeneration[5] and also how VSELs have eluded the scientific community for so long[6]. This article provides an overview of the various approaches used to regenerate pancreas in patients with diabetes, recent advances including our contributions and also a novel approach that may be explored in future.

Islets obtained from the cadavers, foetal tissue or from xeno-source: Success of Edmonton protocol[7] provided the proof of concept for cellular therapy to treat T1DM and current status was reviewed recently[8,9]. Islets isolated from cadavers infused in immuno-suppressed patients with diabetes through the portal vein resulted in quick and sustained insulin production[7]. However, need for alternative source of regulated insulin release is acutely felt due to scarcity of cadaveric islets. Use of foetal islets or those obtained from pigs have associated ethical and immunological concerns. In addition, use of pigs as a source of islets has associated issues like zoonoses.

Stem cells as a source of islets: Use of stem cells including pluripotent stem cells (PSCs), multipotent adult stem cells and progenitors holds lot of promise to treat a variety of diseases. Use of medicines and antibiotics can cure a disease whereas stem cells may be able to replace diseased cells with healthy cells and as a result the patient becomes free of the disease. Both pluripotent and adult stem cells have been used as a source for pancreatic islets. Approval given by US-FDA to study the efficacy and safety of embryonic stem cells derived pancreatic progenitors in T1DM patients is a major step in the field[10].

Pluripotent stem cells: Pluripotent stem cells (PSCs) have the ability to self-renew and differentiate into three germ layers including ectoderm, endoderm and mesoderm, and hence can play an important role in regenerative medicine and cell therapy. PSCs are obtained from the inner cell mass of blastocyst (embryonic stem cells, ES) or from the foetal genital ridge (embryonic germ cells, EG). Human ES cell lines were first reported in 1998 by Prof. Thomson and his group[11] whereas human EG cell lines were reported by Prof. Shamblott in the same year[12]. Technology also exists to derive PSCs from adult somatic cells by reprogramming them to embryonic state using a cocktail of factors (induced pluripotent stem cells, iPS) or by allowing factors present in the oocyte cytoplasm to reprogramme somatic cells (therapeutic cloning). Prof. Yamanaka shared the Nobel Prize for Medicine in 2012 for iPS technology[13] whereas Prof. Mitalipov's group in 2013[14] was the first to derive human ES cell line by somatic cell nuclear transfer (SCNT).

The concept of ES cell therapy is simple and involves differentiation of ES cells (which can be expanded in large numbers *in vitro* due to their immortal status) into pancreatic progenitors for transplantation. The ES cells derived pancreatic progenitors can be packed in immuno-isolatory capsules prior to subcutaneous transplantation under the skin (thus avoid life-long immuno-suppressive therapy) and even if a teratoma forms - it would remain contained in the device and could be safely removed. These encapsulated cells (expected to mature into islets on transplantation) will have the ability to secrete appropriate amount of insulin in a glucose-responsive manner over a period of time. This will be a more physiological approach compared to daily insulin injections and are expected to remain functional over a longer time.

Jiang *et al*[15] observed that 30 per cent of transplanted mice showed reduction in hyperglycaemia on transplanting insulin positive cells, obtained by differentiating ES cells, for over a period of six months. Thus proof of concept for use of human ES cells for diabetes was established, however, the process remains highly inefficient. Schulz *et al*[16] developed a scalable system for producing functional progenitors

and Bruin *et al*[17] improved the differentiation protocol further which resulted in grafts containing >80 per cent endocrine cells and resulted in single hormonal cells expressing either insulin or glucagon or somatostatin in contrast to earlier polyhormonal cells. Kirk *et al*[18] have demonstrated that human insulin is secreted by seven weeks after transplantation of encapsulated pancreatic progenitors and by 20 wk enough human insulin is produced to ameliorate alloxan-induced diabetic symptoms. Endocrine cells that differentiated were monohormonal and insulin was produced in response to a glucose challenge. Thus, impressive progress has been made and the recent approval from US-FDA for a clinical trial using encapsulated cell replacement therapy termed VC-01 from ViaCyte in T1DM patients appears to be very promising[9]. Tabar and Studer[19] have discussed the existing challenges in translating ES based cell therapies to the clinic. These include issues related to huge costs involved, scalability, clinical grade of stem cell products, genetic, epigenetic and safety concerns, *etc*. Although ES/iPS theoretically have the ability to differentiate into functional beta cells but the field has not advanced as expected[20].

Our group after having derived two well-characterized human ES cell lines (KIND1 and KIND2)[21] and studying their propensity[22], carried out studies to differentiate KIND1 ES cells into pancreatic progenitors[23]. Adapting our cell lines to feeder-free state was a big achievement wherein the ES cells initially grown on human foetal fibroblast feeder support were gradually transitioned to feeder-free state and yet the cells maintained their pluripotent characteristics. KIND1 cells have been gradually transitioned into feeder-free state (Fig. 1), expanded in feeder-free state for almost 160 passages and these still maintained a normal karyotype and pluripotent characteristics. Feeder-free KIND1 hES cells were then gradually differentiated into pancreatic progenitors using a modified version of protocol[24]. For differentiation of KIND1 cells into pancreatic progenitors, briefly the KIND1 feeder-free cells at 80 per cent confluence are used for a 16 days differentiation protocol wherein the cells gradually transition from undifferentiated state-definitive endoderm- primitive gut tube- to finally pancreatic progenitor stage[23].

All cells in human body have the same basic genetic information, yet different cells have different morphology and function. Strict epigenetic control by several protein complexes such as polycomb group complexes, trithorax group proteins, histone acetylases, histone deacteylases, DNA methylation enables this feat. Amongst these, polycomb group

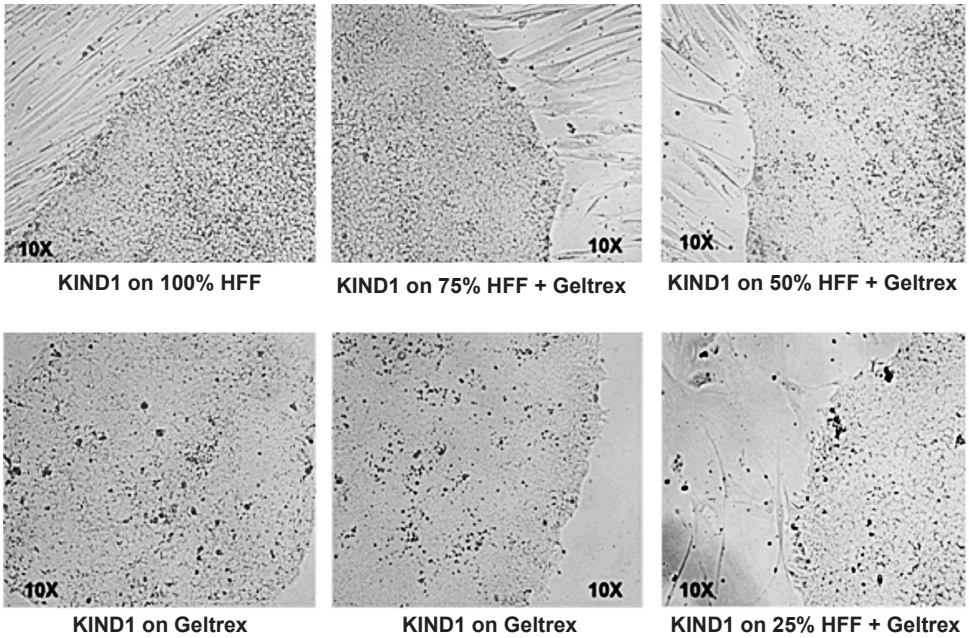

Fig. 1. Establishment of feeder-free culture of human embryonic stem cell. KIND1 cells growing on human feeder fibroblasts (HFF) were transitioned to feeder-free culture system by gradually reducing feeder density while maintaining the concentration of Geltrex (synthetic extracellular matrix).

proteins have received greater attention since these are required during differentiation at embryonic stage and their aberrant expression leads to tumour formation[25,26]. Several polycomb group proteins associate with each other and form large protein complexes called Polycomb Repressive complexes (PRC), two of the well studied PRCs are PRC1 and PRC2. On comparing the epigenetic profile of the pancreatic progenitors obtained after 16 days in culture to adult human pancreas the PRC1 and PRC2 transcripts showed minimal expression in the adult human pancreas compared to high expression in the pancreatic progenitors[23] (Fig. 2). Could this difference in the epigenetic profiles of adult pancreatic cells and the pancreatic progenitors derived from the ES cells prevent long-term benefits after stem cell therapy, remains to be answered. Also these differences could suggest that the differentiated progeny more closely resembles their foetal counterparts rather than the adult (we did not have access to foetal pancreatic tissue sample to do a comparison) as recently concluded by Tabar and Studer[19]. Dimmeler *et al*[27] also conclude that although now we know a lot about stem cells biology, a huge gap needs to be bridged before these cells can be successfully utilized in the clinic. Further studies are required to evaluate whether this epigenetic status of ES-derived progenitors gets ameliorated after transplantation in diabetic mice following further maturation *in vivo*.

Adult stem cells: Besides the pluripotent stem cells as a source of islets, various groups have used adult stem cells like bone marrow and mesenchymal cells to treat diabetes. Two broad directions were followed including *(i)* clinicians directly tested efficacy of autologous stem cells in treating the pancreas in the clinic, and *(ii)* basic scientists studied 'trans-differentiation potential' of mesenchymal stem cells (from various sources) into islets.

Cell therapy using adult stem cells: Bhansali *et al*[28,29] injected autologous bone marrow stem cells directly into the pancreas via the gastrodudenal artery in T2DM and observed certain degree of success. Dave *et al*[30] transplanted autologous adipose tissue transdifferentiated insulin-making cells in two patients with T1DM which resulted in reduced insulin requirement over long term and in a separate study reported that these stem cells were better than bone marrow derived haematopoietic stem cells transplantation[31]. Another group[32] showed that autologous bone marrow stem cells when transplanted through intrahepatic route in

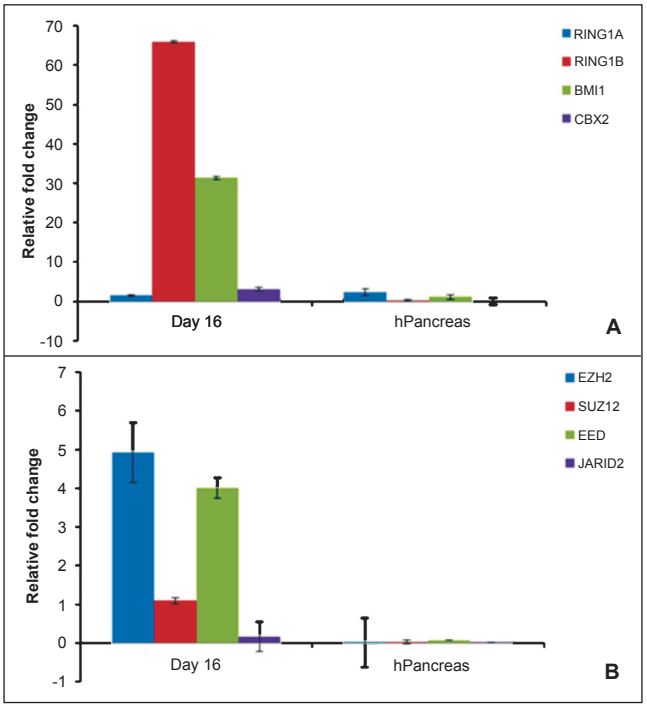

Fig. 2. Comparison of polycomb repressive complexes (PRC) expression in pancreatic progenitors derived from human embryonic stem (hES) cells on day 16 and adult human pancreas. Expression of **(A)** PRC1 (*RING1A, RING1B, BMI1* and *CBX2*) and **(B)** PRC2 (*SUZ12, EZH2, EED* and *JARID2*) complexes was compared by qRT-PCR. Results revealed distinct epigenetic differences of pancreatic progenitors derived from hES cells and adult human pancreas when compared to that of undifferentiated human ES cells used as reference sample; analyzed by $Ct^{\Delta\Delta}$ method. Expression of polycomb group (PcG) proteins in adult human pancreas (normalized to 18S, Ct40) was shown by our group earlier (Ref. 23). These differences may ameliorate after transplantation or possibly are the underlying cause to explain that hES cells differentiate into their fetal counterparts and may thus not be very useful to regenerate adult pancreas. Results are representative of five biological replicates.
[*Source*: Adapted from Ref. 23, Figures 6 & 9, reproduced with permission].

two T1DM patients resulted in increased c-peptide and HbA1c levels and reversed the production of anti-pancreatic islet antibody during 12 months follow up. Liu *et al*[33] have transplanted Wharton's Jelly derived MSCs (through intravenous and intrapancreatic endovascular injection) in 22 T2DM patients. The signs of inflammation, glucose and HbA1c levels were reduced and an improvement of C-peptide levels was observed. They concluded that therapeutic benefit may be due to improved systemic inflammation and/or immunological regulation. However, these are pilot studies showing marginal benefit and are similar to several pilot studies undertaken using autologous bone

marrow stem cells to improve cardiac function. But data are now accumulating that on conducting double blind trials the beneficial effect of stem cell therapy to treat heart diseases may not exist. A meta-analysis of six clinical trials on use of bone marrow stem cell therapy to treat heart disease showed that the effect of therapy on the left ventricular ejection fraction was zero[34].

Basic research using adult stem cells: Several groups have attempted to culture and differentiate MSCs into islets *in vitro*[35-39]. Gopurappilly *et al*[40] used MSCs isolated from the pancreas to differentiate into islets. The foetal islets can be expanded in culture to obtain MSCs[41]. Parekh *et al*[42] used 270 cord blood samples to evaluate the ability of cord blood mononuclear cells to differentiate into islets and concluded that a sub-set of 'pancreas committed cells' existed whose numbers increased after the mice underwent partial pancreatectomy. To conclude, these attempts by various investigators have remained inefficient and the concept remains controversial as it involves de-differentiation followed by re-differentiation into a different lineage since MSCs are mesodermal in origin whereas beta cells are endodermal. Although phenotypic changes are reported of MSCs changing into islets, a robust functional ability of the differentiated islet-like structures has not been demonstrated.

MSCs have also been injected directly in the pancreas and being niche providing cells, these have helped alleviate diabetes symptoms through several mechanisms, such as by improving metabolic control in animal models, counteracting autoimmunity, enhancing islet engraftment and survival or as a source of growth factors and cytokines[43]. It is of interest to note that injecting MSCs not only helps improve pancreatic functions, but also heal associated symptoms like diabetic foot, nephropathy, neuropathy, *etc*. Thus the effect of MSCs appears to be more generalized and most probably is a niche effect rather than true regeneration.

***Regenerating the islets in pancreas*:** Major issue in T2DM is insulin resistance and patients are advised lifestyle changes (diet and exercise) along with insulin sensitizers to improve the sensitivity of muscle, fat and liver for insulin and thus resulting in reduced blood sugar. The sensitivity of various body organs to insulin is reduced and hence insulin requirement is greatly increased but beta cells are unable to secrete. As a result T2DM patients lose their ability to produce insulin and may also benefit if their pancreas could be made functional again by regenerating insulin producing islets from endogenous stem cells. This has led to an interest in understanding endogenous pancreatic stem cells/ progenitors and whether one can target them as a cure for type 2 diabetes. It may thus be possible to replenish the damaged islets in T2DM by stimulating the endogenous stem cells and thus reverse symptoms of diabetes. However, the true identity of pancreatic stem cells remains unresolved[20].

Pancreas is one of the organs besides lung and liver that shows huge potential to regenerate. Regeneration of pancreas occurs successfully even after almost 80-90 per cent of pancreatectomy in mice[44]. The pancreas of mice with streptozotocin induced diabetes can also regenerate after pancreatectomy and reverse diabetes symptoms[45,46]. However, the underlying mechanism how this regeneration occurs, is still controversial. Three schools of thoughts exist including *(i)* reduplication of existing islets, *(ii)* involvement of ductal epithelium, and *(iii)* neogenesis of new islets from stem/progenitor cells. However, the existence and identity of such stem cells/progenitors remain obscure till date as direct proof of their existence is still lacking.

Bonner-Weir's group[47] from the Harvard Stem Cell Institute, USA, has made seminal contributions and developed the concept of ductal epithelium (DE) as a source of pancreatic progenitors that can regenerate adult pancreas after partial pancreatectomy. Pancreatic regeneration is understood to recapitulate embryonic development with a burst of epithelium in the ductal epithelium. They suggest that DE cells undergo de-differentiation to an earlier stage of a progenitor which can differentiate into islets and acinar cells. These progenitors are active during regeneration. However, use of various ductal epithelium specific markers like human carbonic anhydrase II (CAII) promoter[48] or HNF1 β[49] or Sox 9[50] for lineage tracing studies to convincingly show involvement of ductal epithelium in pancreas regeneration has resulted in controversial data. Further studies are required to support the yet controversial ductal origin hypothesis. Kushner *et al*[51] have discussed that if duct cells are not the origin for increased number of islets observed after ductal ligation then what other candidates can produce beta cells so quickly and why they have not yet been identified and reported. They concluded that data generated in next few years will have surprises in the exciting field of pancreatic regeneration.

Xiao *et al*[4,52] have discussed various studies done in support of reduplication of islets as a means for

regeneration versus neogenesis and associated artefacts that could have resulted in controversial results. They used tamoxifen independent INS^{Cre}mTmG mice where all cells are $Tomato^{+}$ (except insulin expressing cells) whereas beta cells are GFP^{+} (green fluorescent protein). They proposed that if any non-islet cell should give rise to the islets – a transient yellow colour will be observed and picked up in an objective manner by flow cytometry. They could not detect any yellow cell by flow cytometry and thus concluded that neogenesis of β cells in the pancreas was a rare event. These results are in agreement with earlier reports[53,54] which also found no evidence of neo-genesis of islets. To conclude from the available literature, despite huge research efforts by several groups worldwide scientific community has not yet been able to throw any light on underlying cellular mechanisms responsible for adult pancreas regeneration. Such knowledge will be helpful to regenerate a pancreas in a patient with diabetes and will have tremendous clinical relevance[20].

We have reported the presence of very small embryonic-like stem cells (VSELs) in adult mouse pancreas[5]. Flow cytometry analysis showed that 0.6 per cent of pancreatic cells are 3-5 μm LIN-/CD45-/SCA+ VSELs. We have further reported that extensive mobilization of octamer-binding transcription factor 4 (OCT-4) positive stem cells occurs into the pancreas after partial pancreatectomy and that these are involved in regeneration[5]. We report the presence of cells that co-express OCT-4 and PDX-1 suggesting that nuclear OCT-4 positive VSELs give rise to PDX-1 positive progenitors which regenerate both acinar and β-cells. We have recently discussed the cause for the existing confusion by various groups and how the VSELs have eluded the scientists because of their very small size[6]. Earlier, VSELs have been reported for the first time in mouse pancreas by Zuba-Surma *et al*[55] and VSELs get mobilized after streptozotocin treatment in mice[56] and also in patients with pancreatic cancer[57].

Similarly, marginal trans-differentiation reported by various investigators while differentiating MSCs into islets could have been because of VSELs which always exist as a sub-group among MSCs[58]. The conclusion made after using 270 cord blood samples by Hardikar's group[45] that a sub-set of 'pancreas committed cells' exists whose numbers increase after the mice undergo partial pancreatectomy was true but the VSELs remained elusive in their study. VSELs are the endogenous pluripotent stem cells responsible for adult pancreatic regeneration and have the potential to treat T2DM. OCT-4 and other pluripotent transcripts are also reported in normal human pancreas[59,60] and during pancreatic cancers[61].

Conclusions

To conclude, there are several available options to regenerate the diabetic pancreas including pluripotent ES/iPS cells, adult stem cells and VSELs. VSELs have an edge over others as these are pluripotent stem cells in adult pancreas; do neither have associated immune-rejection issues nor risk of teratoma formation. However, the controversy surrounding the very existence of VSELs needs to be settled first. These stem cells have remained elusive for decades because of their small size and inadvertently get discarded during processing[6,62]. It is seemingly difficult for scientific community to accept their presence. We have recently reported detailed characterization of cord blood VSELs and shown that these are normal, non-apoptotic, diploid and quiescent cells expressing pluripotent and primordial germ cells specific markers present in the red blood cells pellet after Ficoll-Hypaque centrifugation of cord blood[63]. Moreover, these stem cells regenerate adult pancreas (both acinar cells and islets) and there is no need to culture or expand them in a cyclic guanosine monophosphate (Current Good Manufacturing Practice) facility - rather we need to develop strategies to manipulate the endogenous VSELs to our advantage. There is a need to first arrive at a consensus on the definition of stem cells[62] and then be ready for mid-course corrections to successfully exploit the potential of VSELs in the field of regenerative medicine. This requires extensive brainstorming and support from funding agencies and policymakers to make further progress in the field.

***Conflicts of Interest*:** None.

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