

Chapter 2

Stem Cell Therapy in Diabetes Mellitus

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2.1 Introduction

In 2014, the global prevalence of diabetes mellitus (DM) was estimated to be 9 % among adults aged 18 years and older [1]. Type 1 DM (T1DM) accounts for 5–10 % of diabetic patients. Its pathogenesis involves autoimmune-mediated destruction of the pancreatic islets. Maintenance of appropriate glycemic control is possible using exogenous insulin for life, which imposes a burden on these patients. Transplantation of pancreatic islets or an intact pancreas is an ideal alternative for lifelong treatment. However, the shortage of cadaveric organs and the need for immunosuppression are limiting factors for pancreatic transplantation. Type 2 DM (T2DM) accounts for the majority of diabetic patients, with the highest prevalence in the Eastern Mediterranean region and the Americas. The disease can be initially treated by dietary modifications and oral medication. Eventually, some 27 % of diabetic patients become insulin dependent. Of these, less than half achieve the recommended hemoglobin A1c (HbA1c) level for therapeutic efficacy since exogenous insulin cannot provide the tight glycemic control exerted by pancreas-derived insulin [2].

Recent progress in the field of regenerative therapies provides the potential for the generation of surrogate β -cells, and efforts to engineer insulin-producing cells (IPCs) from stem cells are gaining momentum. Recent studies on IPCs from three

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sources, namely, embryonic stem cells (ESCs), induced pluripotent stem cells (iPS cells), and mesenchymal stem cells (MSCs), derived from a variety of adult tissues will be reviewed in this chapter.

2.2 Embryonic Stem Cells

Cells of embryonic origin have the capacity for rapid replication and the ability to differentiate into cells of all three germ layers (trilineage differentiation). These two characteristics make them an attractive source for the generation of IPCs. Lumelsky and associates reported successful differentiation of mouse ESCs using a five-step protocol [3]. Segev et al. modified the Lumelsky protocol by adding a step of suspension culture at the end of the differentiation protocol [4]. These early reports were challenged by Rajagopal and colleagues, who provided evidence that both the presence of insulin inside the cells and its apparent release are the result of insulin absorbed from the culture medium [5]. Paek and coworkers suggested that insulin release is the result of sequestration of insulin from the culture medium as well as from de novo synthesis [6]. In a series of studies, Baetge and colleagues provided a proof of principle and refined a protocol for the efficient differentiation of human ESCs into insulin-secreting cells. Their differentiation scheme mimicked the in vivo pancreatic development. This was achieved by directing the cells through successive stages toward definitive endoderm, gut-tube endoderm, pancreatic endoderm, and finally pancreatic endocrine lineage [7–10]. The strategy of this group of investigators is to transplant the resulting pancreatic progenitors within an encapsulation device to prevent immunorejection. The grafted cells would undergo further maturation into IPCs under the influence of the in vivo milieu, a process that can take 3–4 months [11]. Using an undifferentiated human ESC line, successful generation of putative IPCs was reported by Pagliuca and associates [12]. These cells share significant functional features within normal human beta cells. Their reported method of differentiation involved a complicated multistep protocol that lasts up to 6 weeks. These authors suggest that using their differentiation protocol, hundreds of millions of glucose-responsive β -cells from human pluripotent stem cells can be produced. It is clear that important progress in differentiating ESCs into IPCs has been achieved. However, the use of embryonic cells suffers from two drawbacks: their teratogenicity and immunogenicity. These two problems could be contained if such cells are transplanted within an encapsulation device.

2.3 Induced Pluripotent Stem Cells

Yamanaka and his groups were the first to prove that by forcing the expression of a small number of factors, terminally differentiated cells could revert back to a pluripotent state [13, 14] and were termed *induced pluripotent stem cells* (iPS cells).

Initial derivation of iPS cells utilized retroviral-mediated introduction of Oct3/4, Sox2, Klf4, and c-Myc (Yamanaka factors). Later, nonviral methodologies were introduced. Repeated transfection of plasmids containing the Yamanaka factors resulted in the production of iPS cells without evidence of plasmid integration [15, 16]. The iPS cells generated from somatic cells are expected to resolve problems that pertain to embryonic cells. The use of embryonic cells has been limited to certain established clones; accordingly, immunorejection is considered a major obstacle for cell therapy. In contrast, patient-derived iPS cells would theoretically not suffer immunorejection since they are autologous. However, the efficiency of their generation remains low [17]. Moreover, the formed iPS cells show unlimited proliferative activity and form teratomas upon transplantation [18]. They also carry epigenetic memory characteristic of the somatic cell of their origin. This favors differentiation along lineages related to the donor cells [19].

The pluripotency of generated iPS cells provides a potential for their differentiation to IPCs. Tateishi et al. were probably the first to report the possibility of generating insulin-secreting isletlike clusters from iPS cells derived from human skin fibroblasts [20]. Using a three-step differentiation protocol, iPS cells derived from mice fibroblasts were differentiated into IPCs by Alipio and associates [21]. These cells were able to reverse hyperglycemia in diabetic mouse models. Zhu and colleagues developed a four-stage protocol to generate IPCs from rhesus monkey iPS cells. The resulting cells could secrete insulin in response to glucose stimulation, and when they were transplanted into diabetic mice, the blood glucose levels were reduced in 50 % of the treated animals [22]. Jeon et al. generated iPS cells from Non obese diabetic (NOD) mouse embryonic fibroblasts and from NOD mouse pancreatic epithelial cells. They applied a directed differentiation protocol to induce the formation of functional pancreatic beta cells. They found that the iPS cells derived from NOD mouse pancreatic epithelial cells differentiated more readily into IPCs. Transplantation of these cells in diabetic mice could normalize their blood glucose levels [23]. In a more recent report, human iPS cells derived from both fetal and adult human tissues were differentiated *in vitro* into pancreas-committed cells. At the end of *in vitro* differentiation, approximately 5 % of cells became insulin positive. When transplanted into immunodeficient mice, the transplanted cells lost their insulin secretion capacity in response to glucose stimulation. Histology of the graft demonstrated a mixed population of cells containing pluripotent, neuronal, and mature pancreatic cells [24].

It is abundantly clear that the utilization of iPS cells to form IPCs requires further refinements and optimization before their application can be clinically meaningful.

2.4 Mesenchymal Stem Cells

Earlier studies by Friedenstein and colleagues reported that bone marrow stroma could generate bone, fat cells, and cartilage following heterotropic transplantation in mice [25]. This suggested the existence of non hematopoietic bone marrow

precursor cells with skeletal and adipogenic potential. The notion of a stromal stem cell was proposed subsequently by Owen [26]. The term *mesenchymal stem cells* (MSCs) was popularized by Caplan to refer to plastic-adherent cell preparations isolated from a variety of tissues [27]. Recently, leading investigators of mesenchymal cell therapy concluded that convincing data to support the “stemness” of these unfractionated plastic-adherent cells are lacking [28]. Therefore, the term *mesenchymal stromal cells* has been suggested, allowing the abbreviation “MSCs” to be maintained. Several independent studies have demonstrated that MSCs can differentiate not only into mesodermal but also ectodermal and endodermal lineages [29]. Based on these findings, the term *multipotent mesenchymal stromal cells* appears to be the most scientifically accurate descriptor of this plastic-adherent population. The term *mesenchymal* is maintained to imply the origin, but not the differentiation potential, of these cells [30]. The International Society for Cellular Therapy proposed three criteria to define MSCs [30]. First, MSCs must be plastic adherent when maintained in standard culture conditions using tissue culture flasks. Second, 95 % of the MSC population must express CD105, CD73, and CD90 as measured by flow cytometry. In addition, these cells must lack expression (≤ 2 %) of CD45, CD34, CD14, and HLA class II. Third, the cells must be able to differentiate into osteoblasts, adipocytes, and chondrocytes under standard culture in vitro differentiating conditions.

MSCs can be derived from a variety of human tissues and have a high capacity to replicate. They are easy to cultivate and expand and can maintain their multilineage potential following prolonged culture conditions [31]. In addition they are nonteratogenic and their utilization is free of any ethical consideration. All of these reasons have rendered them a good tool for use in regenerative medicine, including in potential therapeutic use for DM.

MSCs derived from different sources were coaxed using different approaches to differentiate into IPCs. The bone marrow [32–34], adipose tissue [35], umbilical cord, umbilical cord blood [36, 37], fibroblasts [20], endometrium [38], and liver cells [39] are among several tissues that are rich in MSCs. Of these, the bone marrow and adipose tissue offer distinct advantages in view of their availability, abundance, and the extent of their documentation in the literature. To this end, two approaches were used for their differentiation into IPCs: genetic manipulation or directed differentiation. Transfection with genes important in pancreatic development was reported by several investigators. Karnielli et al. transfected human bone marrow stem cells with a virus vector carrying a rat PDX-1 gene. The extent of differentiation of these cells toward the β -cell phenotype was evaluated. The authors reported that the treated cells expressed all four islet hormones but lacked the expression of NeuroD-1. Cell transplantation into streptozotocin (STZ)-induced diabetic immunodeficient mice resulted in their further differentiation, including the induction of NeuroD-1 and reduction of hyperglycemia [34]. Porcine bone marrow stromal cells were electroporated with an insulin-expressing plasmid vector. When these cells were engrafted in the liver of STZ-induced diabetic pigs, partial but significant improvement in hyperglycemia was observed [40]. For directed differentiation, many protocols were evaluated using culture media rich in glucose [41, 42]. Initial experiments used cells of murine origin

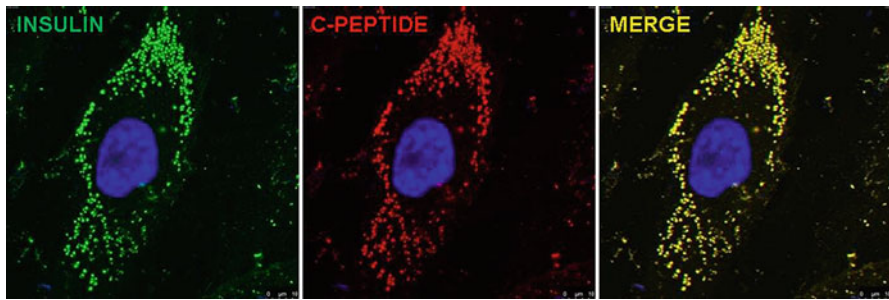


Fig. 2.1 Immunofluorescence staining of differentiated HBM-MSCs (a selected field). (a) Positive staining for intracytoplasmic insulin granules (*green*) with counterstaining for DAPI (*blue*). Positive staining for c-peptide (*red*) with counterstaining for DAPI (*blue*). Electronic merge of insulin and c-peptide staining. The coexpression of insulin and c-peptide (*yellow*) was detected in the same cells

[43–45]. Subsequently, MSCs derived from human tissues were tried [23, 35, 46]. The early reports demonstrated variable degrees of success but were met with skepticism since it was argued that MSCs should not differentiate toward an endocrine pancreatic lineage.

In our laboratory [32], bone marrow cells were obtained from three adult diabetic and three nondiabetic volunteers. MSCs were isolated, expanded, and differentiated using a three-stage protocol. Cells were cultured in a glucose-rich medium containing several activation and growth factors. Initially, mercaptoethanol was used to induce the cells toward a pancreatic endocrine lineage. Subsequently, nonessential amino acids, basic fibroblast growth factor, epidermal growth factor, and B27 supplement were added. Finally, activin A and nicotinamide were supplemented. At the end of differentiation, approximately 5 % of cells were positive for insulin and c-peptide by immunofluorescence. Insulin and c-peptide were coexpressed by the same cells (Fig. 2.1). Electron microscopy with nanogold immunolabeling demonstrated the presence of c-peptide granules in the rough endoplasmic reticulum. The differentiated cells expressed transcription factors and genes of pancreatic hormones similar to those of pancreatic islets. Furthermore, there was a stepwise increase in human insulin and c-peptide release in response to increasing glucose concentrations. Transplantation of these cells into diabetic nude mice resulted in control of their diabetes. The sera of the treated mice contained human insulin and c-peptide with negligible levels of mouse insulin. When the kidneys bearing the transplanted cells were removed, rapid return of diabetes was noted. In summary, evidence was provided that MSCs can indeed be differentiated into IPCs. Nevertheless, two observations remained to be addressed. First, improvement of the yield of IPCs following directed differentiation of human bone marrow-derived mesenchymal stem cells (HBM-MSCs) is needed. Second, an explanation of the ability of transplanted cells to cure diabetic nude mice in spite of the modest yield of IPCs *in vitro* is also required.

In a subsequent study, we compared the efficiency of the original protocol in which mercaptoethanol was used for the induction of differentiation of HBM-MSCs into IPCs with two other agents: conophylline and trichostatin [47]. The yield of functional IPCs was again modest and comparable among the three protocols ($\approx 3\%$). This is in agreement with the *data* of other investigators who reported that the proportion of IPCs at the end of *in vitro* differentiation was small irrespective of the employed protocol. In view of its simplicity and the short period required for its completion, only 10 days, the trichostatin-based protocol is currently our traditional method for directed differentiation of HBM-MSCs into IPCs. Several laboratories have also reported that, although the proportion of IPCs generated *in vitro* from MSCs was meager, they could induce euglycemia when the cells were transplanted into diabetic nude mice [48, 49]. Without providing clear evidence, it was suggested that this was the result of further maturation of the implanted cells *in vivo*. To confirm this finding, we carried out a series of experiments in our laboratory [50]. HBM-MSCs were obtained from three insulin-requiring type 2 diabetic patients. Following expansion, cells were differentiated according to a trichostatin-A/GLP protocol. One million cells were transplanted under the renal capsule of 29 STZ-induced diabetic mice. Mice were euthanized 1, 2, 4, and 12 weeks after transplantation. The IPC-bearing kidneys were immunolabeled, the number of IPCs counted, and the expression of relevant genes determined. The diabetic animals became euglycemic 8 ± 3 days after transplantation. The percentage of IPCs from the harvested kidneys increased gradually to reach a peak of $\approx 18\%$ at 4 weeks after transplantation without a substantial change thereafter (Fig. 2.2). Relative gene expression of insulin, glucagon, and somatostatin showed a similar increase. We concluded that the ability of the transplanted cells to induce euglycemia was due to an increase in the numbers of IPCs. It is reasonable to assume that the *in vivo* milieu contains factors that promote the maturation of the transplanted cells. It was reported that the source of these factors can be from the regenerating pancreas after it had sustained a toxic or traumatic injury [51]. It was shown that cytosolic extracts from the regenerating pancreas have the potential to initiate neogenesis in STZ-induced diabetic animals [52]. An extract obtained from a regenerating pancreas 2 days after 60 % pancreatectomy was utilized with success for differentiation of rat mesenchymal cells into IPCs [53]. Further studies to identify the factor(s) secreted during pancreatic regeneration can provide an important tool for achieving the efficient differentiation of HBM-MSCs into IPCs.

It is clear that only a subset of MSCs is capable of trilineage differentiation [54]. The identification, sorting, expansion, and subsequent differentiation of this subpopulation can result in the production of sufficient IPCs with adequate functional capacity. Previous reports by Catherine Verfaillie's group described a culture system for MSCs that favors the selection of a subpopulation of primitive cells referred to as *multipotent adult progenitor cells* (MAPCs) [55]. A variety of other cells derived from postnatal tissues that demonstrated pluripotency were more recently reported: unrestricted somatic stem cells (USSCs) [56], marrow-isolated adult multilineage-inducible cells (MIAMIs) [57], and very small embryonic-like (VSEL) stem cells [58]. However, all of them were associated with controversies regarding lack of

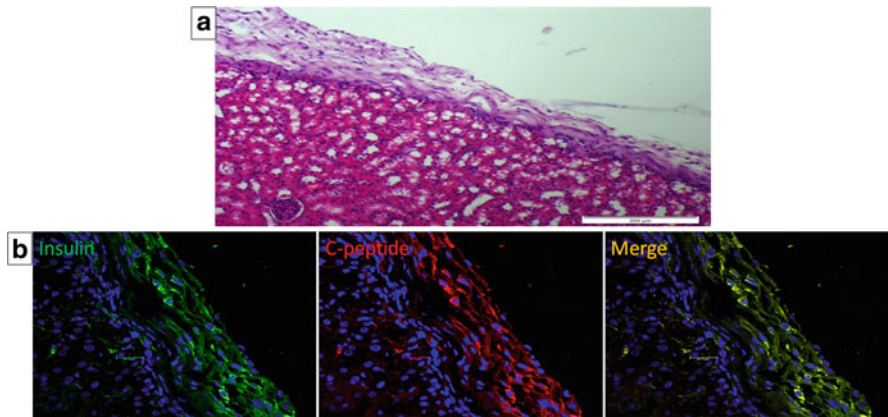


Fig. 2.2 Histology of IPC-bearing kidneys harvested from mice 4 weeks after transplantation. (a) Hematoxylin and eosin staining revealed the implanted cells beneath the renal capsule. (b) Positive immunofluorescence staining for insulin (*green*) and c-peptide (*red*). Electronic merge (*yellow*) reveals the coexpression of insulin and c-peptide in the same cells

reproducibility and skepticism. The intermediate filament protein nestin has been detected in several cellular phenotypes during embryonic and adult life. It was proposed that the expression of nestin may reflect the multipotential and regenerative abilities of cells [59]. Kabos et al. described a method for isolating nestin-positive cells from adult bone marrow [60]. Using this method, successful differentiation of nestin-positive subset of bone marrow-derived pancreatic endocrine cells was achieved by Milanesi and colleagues [61]. However, superiority of this method over the use of unfractionated cells in terms of the number or functionality of the generated IPCs was not shown. Recently, Kuroda and colleagues isolated what they defined as multilineage-differentiating stress-enduring (Muse) cells cultured from skin fibroblasts or bone marrow stromal cells [62]. These cells were positive for both CD105, a mesenchymal cell marker, and stage-specific embryonic antigen-3 (SSEA-3), a human pluripotency marker. Muse cells were indistinguishable from other MSCs in adherent culture, but when they are transferred to suspension culture, they form characteristic cell clusters that are capable of self-renewal as well as differentiation into all three germ layers. To our knowledge, the differentiation of these cells into IPCs has not been reported yet.

2.5 Mesenchymal Stem Cells and Diabetic Complications

Uncontrolled or poorly controlled DM promotes the development of serious complications. These result essentially from vascular pathologies. Microvascular affections manifest as retinopathy, nephropathy, and debilitating neuropathies.

Macrovascular involvements lead to accelerated cardiac disease, sexual dysfunctions, and diabetic foot ulcers.

MSCs have the ability to migrate and home in injured tissues, where they act by secreting trophic factors and paracrine mediators, leading to their regeneration. As a result, research efforts are now directed not only to generating IPCs but also to use unmodified MSCs in the management of serious diabetic complications. Experimental evidence shows that MSCs can reverse the manifestations of diabetic neuropathy [63] and retinopathy [64]. MSCs were utilized with success in the treatment of rabbit ulcer model [65]. It was also reported that MSCs ameliorated podocyte injury and proteinuria in a rat model with type 1 diabetic nephropathy [66]. A possible role for MSCs in the regeneration of intervertebral disk was suggested by Huang et al. [67]. Given the capacity of MSCs to home to damaged tissues, their possible role in the management of infertility or reproductive disorders was also reported [68]. Out of 86 diabetic patients, treatment with bone marrow-derived MSCs promoted ankle nonunion healing in 70 [69].

2.6 Concluding Remarks

To establish persuasive proof that a certain type of stem cell has been successfully differentiated into IPCs, Calne and Ghoneim [70] defined the following criteria: (1) coexpression of insulin and c-peptide by the same cells, (2) demonstration of insulin storage granules, (3) identification of specific gene expression similar to those of pancreatic β -cells, (4) stepwise increase in insulin and c-peptide release as a function of increasing glucose concentration in vitro, (5) cure of hyperglycemia following cell transplantation in diabetic animals, and (6) prompt return of diabetes when these cells are removed. It is abundantly clear that stem cells, embryonic cells, and induced pluripotent or mesenchymal stem cells met these criteria at the experimental level. For the translation of these research findings to a clinical application, additional questions need to be answered: How many functioning cells are needed per kilogram body weight? How long will these cells remain functioning? What is the optimal site for their transplantation?

In August 2014, the US Food and Drug Administration (FDA) approved an Investigational New Drug (IND) application for the treatment of patients with type I DM to be carried out by a biotechnology company in San Diego, California (ViaCyte). Pancreatic progenitor cells derived from a human embryonic cell line will be transplanted within a device to prevent allogeneic rejection. Their strategy depends on spontaneous maturation of the grafted progenitor cells into IPCs in the body. It is a first step and will not be the last. Medical history indicates that innovations are always incremental. The potentials of Muse cells are great owing to their pluripotency, the lack of teratogenicity, and the possibility of their use in an autologous fashion. The caveat in using these cells is that, with expansion, they lose their pluripotency marker and tend to differentiate into cells of their tissue of origin.

Meanwhile, the potential applications of unmodified MSCs for the treatment of diabetic complications are ever growing.

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