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## Review Article

# *In vitro* (re)programming of human bone marrow stromal cells toward insulin-producing phenotypes

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Diabetes mellitus is a chronic disease with great social and economical impact. In all its forms, it affects nearly 200 million people worldwide (1). Type 1 diabetes (T1D) represents 10% of all cases. Because of the increase in obesity in developed countries, the prevalence of type 2 diabetes (T2D) has been rising very rapidly, affecting children and adolescents.

T1D and advanced T2D are caused by a progressive loss of functional pancreatic β-cell mass within the pancreatic islets. The extraordinary results of the Edmonton protocol have shown that human pancreatic islet transplantation can normalize glycemic control of insulin-dependent diabetic patients (2). Nevertheless, this therapeutic option does not represent a significant clinical benefit for all diabetic patients; the demand of islets for transplantation is very high and human donor pancreas for isolation of islet grafts is limited. Furthermore, transplants do last no longer than 2 yr and are accompanied by significant side effects as a consequence of lifelong aggressive immunosuppression (3). Therefore, intensive search for new sources for β-cell replacement has been undertaken. Regeneration of existing mature β-cells and replacement of insulin-producing cells are current research lines that could possibly solve the problem of islet shortage. Differentiation of embryonic stem (ES) cells has been thoroughly investigated; however, results are not yet satisfactory (4–6). Adult stem/progenitor

cells of pancreatic and extrapancreatic origin have been demonstrated to differentiate *in vitro* into insulin-producing phenotypes (7).

Bone marrow stromal cells (BM-MSC) display adult stem cell character (8). Despite their mesodermal origin, the capacity of BM-MSC to restore β-cell function has been demonstrated by several groups (9, 10). Notwithstanding, the fate and function of *in vitro* cultivated BM-MSC and *in vivo* transplanted BM-MSC appear to diverge.

This article focuses on recent experiments on BM-MSC as a suitable source for β-cell replacement and its putative therapeutic potential in diabetes mellitus.

## Potential sources for cell-based therapy in diabetes mellitus

### ES cells

ES cells are non-committed cells with infinite self-renewal capacity, which, under specific conditions, are able to differentiate into cells of any tissue. It has been shown that ES cells may provide an unlimited supply of pancreatic β-cells (11). In the past 2 yr, important steps have been taken toward differentiation and isolation of endodermal pancreatic precursor cells from rodent and also human ES cells with subsequent generation of insulin-producing phenotypes (4–6). Regrettably, differentiation efficacy is low and, so far, no glucose-responsive insulin secretion was observed in these phenotypes.

In spite of the enormous potential of ES cells, major ethical concerns regarding the harvesting of human ES cells, together with the associated tumorigenic risk of developing teratocarcinoma (12), have been delaying the breakthrough of ES cells for the development of new forms of regenerative medicine. Nevertheless, in the diabetes field, ES cell investigation has been crucial to uncover developmental steps of human pancreatic endocrine cells *in vitro* because almost all data available are based on animal models.

### Adult stem cells within and outside the pancreas

Identification, isolation, and *in vitro* proliferation of stem/progenitor cells within the endocrine pancreas might represent an ideal solution for β-cell replacement in T1D. Previous experiments *in vitro* and/or *in vivo* described the existence of precursor cells with potential to differentiate into insulin-producing phenotypes in the pancreatic islets (13, 14), ducts (15), and acini (16), as well as within the adult and fetal pancreas. Both mechanisms of bona fide proliferation and neogenesis have been implicated in the generation of new β-cells in response to specific stimuli. Yet, the existence of a 'true' pancreatic endocrine stem cell has been strongly questioned. Genetic lineage tracing studies have demonstrated that physiological β-cell regenerative processes occur through replication of existing mature β-cells and not via differentiation of pancreatic progenitor cells (17). This conflicts with recent studies that corroborate the existence of pancreatic precursor cells (18, 19). Using a unique pancreatic injury model, Xu et al. has convincingly demonstrated the *de novo* generation of β-cells from pancreatic progenitor cells through reactivation of neurogenin 3, a marker of pancreatic endocrine precursor cells in the developing pancreas (20).

After intensive investigation in the last few years, it is now well accepted that populations of adult cells residing in a specific tissue are not necessarily committed to a specific cell lineage. Despite discussion on mechanisms and pathways responsible for differentiation (neogenesis, differentiation, transdifferentiation, or cell fusion) (21), *in vitro* and *in vivo* experiments have shown that adult stem cells from different origins could be induced to replace or regenerate injured tissues by overwhelming germ layer boundaries (7).

Extrapancreatic stem/progenitor cells from liver (22, 23), central nervous system (24), and bone marrow (BM) (9, 10) have been described to express and produce insulin *in vitro*, when subjected to specific biological conditions or genetic manipulation strategies. While some of these studies claimed the potential of differentiated cells to ameliorate hyperglycemia in diabetic animal models, the *in vitro* insulin secretion is limited and glucose-sensing response is reduced compared with native β-cells, which means that complete differentiation process into mature β-cell-like phenotypes could not be achieved *ex vivo* and the mechanisms of *in vivo* amelioration are unexplained yet.

Umbilical cord-derived mesenchymal stem cells (UC-MSC) have been suggested as an attractive source for the cell-based therapy of diabetes mellitus. In previous experiments, isolated UC-MSC have been found to reduce insulitis and ameliorate glycemic control when transplanted into T1D mouse models (25), but no study has so far reported differentiation of UC-MSC into insulin-producing phenotypes.

Studies on peripheral blood cells with the capacity to transdifferentiate into insulin cell types are few and could never be reproduced (26). BM-derived hematopoietic stem cells (HSC), which have been a valuable source for the cellular treatment of several hematological diseases, showed controversial results in diabetic mice models (27, 28). The observation that HSC differentiate *in vivo*, into insulin-positive cells, was never replicated (28). Moreover, it has been suggested that the most probable effect of infused HSC is to induce regeneration of the injured islets in diabetic pancreas (27).

In humans, only one clinical trial has described a successful therapeutic outcome after autologous non-myeloablative HSC transplantation in newly diagnosed T1D. Indeed, improved peak stimulated C-peptide

levels, decreased glutamic acid decarboxylase (GAD) antibodies, and prolonged insulin independence were observed in a significant number of patients (29). Because of the administration of immunosuppressive agents and antithymocyte globulin to these patients, it is impossible, in a context of autoimmune disease, to evaluate the beneficial effects of HSC transplantation vs. immunomodulation. Moreover, it is questionable if such an intervention with well-known acute and long-term complications is ethically acceptable in a treatable disorder like T1D.

## Mesenchymal stem cells

Mesenchymal cells are part of the 'stroma', which, in turn, corresponds to the supporting framework of each tissue or organ (Fig. 1). Stromal cells ensure major support functions, including a three-dimensional structure (fibroblasts, collagen, and vascular cells), nutritional source (blood supply), and tissue repair (endothelial cells, immune cells, mesenchymal cells, growth factors, and cytokines). These properties have raised the interest of investigators on stromal cells for regenerative medicine.

### Mesenchymal stromal cells are multipotent cells

The multilineage differentiation capacity of mesenchymal stromal cells (MSC) has been initially described in the BM (8). BM includes at least two distinct populations of cells, both with high plasticity: hematopoietic $CD34^+$ stem cells (HSC) –the precursors of all mature blood cells (30) –and mesenchymal $CD34^-$ stem cells (BM-MSC). BM-MSC are a specific population within marrow stromal cells that have been shown to differentiate into mesodermal lineages, including fat, bone, cartilage, muscle and also toward epithelial, neuroectodermal, and endodermal phenotypes. More recently, cells with MSC properties have been identified in adipose tissue and umbilical cord, placenta, and synovium (31).

### Characteristics of BM-MSC

Because of their migration and homing capacity, mesenchymal cells play a fundamental role in embryonic morphogenesis, tissue repair, and regeneration (32). In BM-MSC, expression of several chemokine receptors, such as Cxrc4 receptor, for stem cell factor 1 strongly suggest this property (33). Furthermore, extensive *in vitro* studies have demonstrated that BM-MSC can exert profound immunosuppressive effects via modulation of cellular immune pathways (34–36). Additionally, the immunophenotype of BM-MSC ($MHCI^+$, $MHCII^-$, $CD80^-$, $CD86^-$, and $CD40^-$) is considered non-immunogenic. This feature may allow for allogenic transplantation of the cells with no need for immunosuppression. On the other hand, the stromal supporting nature of MSC offers an additional advantage for maintenance of the grafts or regeneration of injured tissues.

### Isolation of BM-MSC

According to the International Society for Cellular Therapy, MSC can be distinguished by *in vitro* plastic adherence, self-renewal, specific surface markers, and

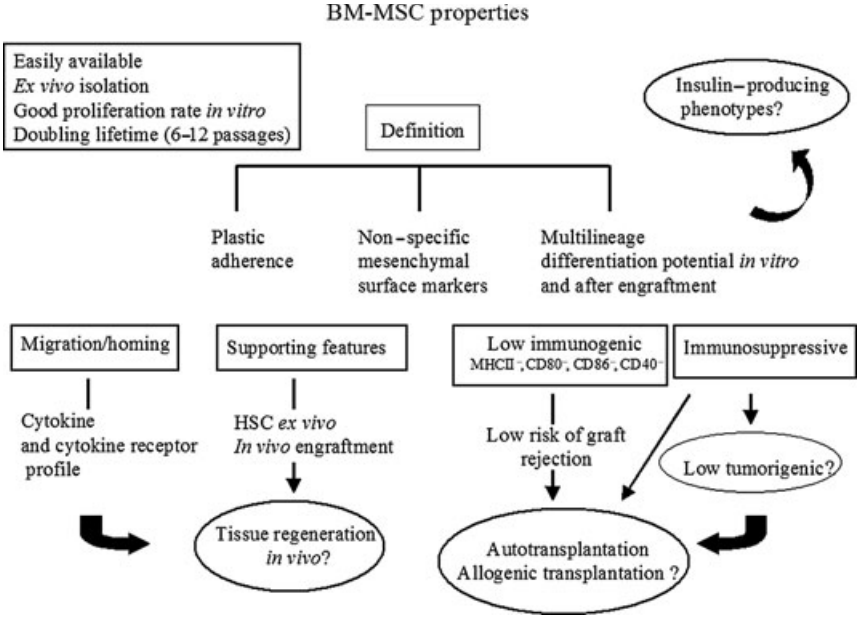


*Fig. 1.* The bone marrow-derived mesenchymal stem cell (BM-MSC) properties: most important features of BM-MSC, which renders these cells very attractive for cell-based therapies and in particular for β-cell replacement in type 1 diabetes..

differentiating capacity into multiple mesenchymal lineages following engraftment (37). Yet, so far, there is no consensus marker panel that allows for prospective identification of MSC from the *in vitro* marrow stromal cell population. Recently, it has been reported that neuro ganglioside GD2 surface marker is expressed in BM-MSC and not in other marrow stromal cells (38). Moreover, SSEA-4, a glycolipid antigen marker of human ES cells (39) as well as the nucleolar protein nucleostemin are suggested as adult MSC markers (40).

### Are adult BM-MSC a promising source for β-cell replacement?

*In vitro and in vivo reprogramming of adult cells.* Reprogramming adult somatic cells implicates induction or repression of key regulatory genes, which are able to switch cells from one lineage into another or to dedifferentiate a mature phenotype back to a more primitive state to obtain pluripotency.

In recent pioneering experiments, mouse and human fibroblasts have been successfully reprogrammed into ES-like stem cell lines. These induced pluripotent stem cells (iPS) were generated by *in vitro* retroviral transduction of four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) that completely modified the epigenetic signature and phenotype of these cells (41, 42). Using the same techniques, murine liver and stomach cells have also been reprogrammed into iPS cells (43).

Moreover, reprogramming of adult exocrine pancreatic cells into functional β-cells was recently achieved by re-expressing a combination of key regulatory genes (Ngn3, Pdx1, and MaFA) in living adult animals. A great advantage of this *in vivo* strategy was to maintain the viral transduced exocrine cells in their native environment, which may favor maturation and survival of newly reprogrammed β-cells (44).

Because of their multipotent properties, MSC may have an almost unlimited differentiation potential once they have been reprogrammed. This might be achieved by specific culture conditions, genetic induction of key transcriptions factors, or more recently through epigenetic modifications.

## Reprogramming BM-MSC into insulin-producing phenotypes

In recent years, an increasing body of evidence suggests that BM-MSC have the potential to differentiate *in vitro* into insulin-producing cells in mouse (9, 10) and human systems (45, 46) and to revert hyperglycemia at least in diabetic mouse models (9, 10, 45). Initial results have been achieved by *ex vivo* expansion of plastic adherent BM-MSC and prolonged endocrine cultivation conditions (9, 10). More recently, the endocrine β-cell-like phenotype was obtained by forced expression of a pancreatic endocrine key regulator, Pdx-1, in primary BM-MSC, with higher differentiation efficiency (45). Furthermore, epigenetic modification could induce differentiation of mouse BM-MSC into insulin-producing cells, even in a glucose-sensing manner (47). Nevertheless, efficacy of BM-MSC differentiation is low and highly variable. The most important limitation at the current state of knowledge is the insulin secretion capacity of these cells, which is far from native β-cells. While several studies have demonstrated the potential of endocrine-differentiated BM-MSC to produce and secrete insulin in a glucose-sensing manner, insulin amount is still significantly lower compared with mature β-cells (9, 10, 45, 47).

This might be related to donor-specific variability, heterogeneity of MSC preparations, and non-optimized differentiation protocols. Also, there might be unknown BM-MSC populations with suitable endocrine commitment to be found. In an attempt to overcome such issues, we used a well-characterized cell line, the telomerase-immortalised human adult bone marrow-derived mesenchymal stem cell line (hMSC-TERT), as a human BM-MSC model to investigate the molecular mechanisms of endocrine differentiation in MSC (Limbert C, Päth P, Ebert R, Rothhammer V, Kassem M, Jakob F, Seufert J, unpublished data). These cells were able to induce insulin in response to specific culture conditions or via ectopic expression of two major pancreatic endocrine genes, Ngn3 and/or Pdx1. Specifically, our data indicate that Ngn3 transcription factor is involved in Pdx1 and insulin expression in hMSC-TERT, thus mimicking the pancreatic endocrine transcription factor cascade in the developing pancreas. Still, insulin secretion was reduced and no response to glucose stimulation was observed, which indicates reduced endocrine differentiation capacity. Although hMSC-TERT display 'endodermal prepatterning' features, molecular tools for the glucose-sensing machinery are missing in this cell line. *In vivo* experiments could favor the endocrine maturation of modified hMSC-TERT. Moreover, a different combination of key regulatory genes might be more adequate to obtain functional β-cell-like phenotypes out of this population.

Anyhow, it is well accepted that independently of the protocol and of the MSC population or donors, reprogramming BM-MSC into functional insulin-producing phenotypes *in vitro* requires several important biological steps presented in Table 1.

## Transplantation of adult BM-MSC into diabetic mouse models

The direct transplantation of donor BM-MSC into diabetic mice have convincingly demonstrated that the major effect of BM-MSC infusion is to enhance the number of endogenous mouse β-cells with increase of mouse insulin and amelioration of

Table 1. Major biological steps for reprogramming BM-MSC into functional insulin cell-types

| |
|---|
| 1. Gene expression markers for: |
| (i) Endodermal patterning of MSC: ABCG2, Cxcr4, Thy-1, Foxa2, Sox17, Isl1, Hlxb9 |
| (ii) Endocrine precursor cells: Ngn3, NeuroD, Nkx6.1, Pax4 |
| (iii) Mature β-cell |
| Key regulators of insulin expression-Pdx1 and Maf-A |
| Proinsulin (Ins) |
| Glucose transporter-Glut2 |
| Glucose sensing-machinery- Glucokinase (Gck) |
| 2. Protein synthesis |
| Insulin and/or C-peptide |
| 3. Insulin secretion in response to glucose challenge *in vitro* |
| 4. Improvement of hyperglycemia in diabetic animal models and/or humans after engraftment of insulin-producing phenotypes |
| 5. Excision of the implanted cells leads to hyperglycemia in the diabetic models |

Expression of early endodermal markers in isolated adult BM-MSC indicates the presence of suitable BM-MSC cell sub-population. After the endocrine differentiation process, expression of major regulatory transcription factors is mandatory to recognize endocrine precursor phenotypes or mature β-cells. The function of insulin-producing phenotypes is assessed by the expression of insulin gene, specific glucose transporter and glucose sensing factors, as well as production, storage, and secretion of insulin protein in a glucose-dependent manner. *In vivo*, these cells should revert hyperglycemia in overt diabetic animal models and excision of the engrafted cells triggers hyperglycemia.

hyperglycemia (48, 49). In contrast to previous findings (28), differentiation of donor BM-MSC into insulin-producing cells seems to be rare. Interestingly, transplantation of *in vitro* endocrine-differentiated BM-MSC into streptozotocin injured non-obese diabetic mice increases insulin and C-peptide levels as well as improves glycemic control. Nevertheless, as shown by hyperglycemia after excision of the transplant, there is no regeneration of endogenous mouse β-cells (10, 45). These observations strongly suggest that, *in vivo*, fate and function of endocrine-differentiated BM-MSC are distinct from that of directly infused BM-MSC. Yet, both can either revert or ameliorate diabetes.

At this point, some reflection should be made to establish which mechanism is more suitable to be adopted in cell-based strategies: cell reprogramming with consecutive cell replacement or *in situ* induction of β-cell regeneration.

## Other promising approaches to cure T1D mellitus

*In vivo* restoration of β-cell mass might prove beneficial for the reversal of hyperglycemia, but protection of the expanded β-cells from ongoing autoimmune destruction is equally important to cure T1D mellitus.

The efficacy of immunosuppressive agents such as prednisone, azathioprine, and cyclosporine to suppress T lymphocytes in T1D patients has been thoroughly evaluated (50, 51). Although slower decline in C-peptide levels could be observed in these patients, withdrawal of the drugs led to progression of β-cell mass destruction, which would implicate lifelong immunosuppression with all known chronic complications.

More targeting immunomodulatory approaches have been developed and are now on early phase clinical trials. Treatment with either a peptide of the heat-shock protein 63, Diapep277® (Andromeda Biotech Ltd.'s, Yavne, Israel), an autoantigen with immunomodulatory properties, or anti-CD3 antibody has been effective in reducing insulitis and insulin requirements in newly diagnosed T1D mice and humans (52, 53). Results of a most recent clinical trial revealed that vaccination with two dose of recombinant GAD65 peptide, in early diagnosed type 1 diabetics (< 6 months), induces preservation of residual β-cell mass. This seems to result from stimulation of an antigen-specific T-cell population and modulation of B-cell memory (54). Similar to anti-CD3 treatment, GAD vaccination slowed the decline of stimulated C-peptide level, conferring a metabolic benefit after 18 months of trial (53). In contrast to anti-CD3 therapy, no reduction in insulin dose requirements was observed in treated patients and no side effects were detected.

Yet, none of the described immunomodulatory approaches has been sufficient for inducing insulin independence.

## Conclusion and perspectives

While cell-based therapy has still a fairly long way to go in order to deliver a full response to T1D, there is still room for exogenous insulin strategies developments. Using nanoparticle systems, new concepts of glucose-sensing insulin release are being tested on diabetic animal models. SmartInsulin aims at being a once-a-day subcutaneous insulin formulation liberated in a glucose-regulated manner. Then again, the existence of molecular signals other than glucose that could unnecessarily release insulin into the circulation must be certified to avoid dangerous bursts of insulin. Whereas Smart particle delivery systems might represent a relevant alternative in the treatment of T1D, it does not comprise the key to insulin independence.

Because of their intrinsic properties, MSC display great potential to be used as a real alternative source for cellular therapy in many degenerative diseases, including T1D.

Much has been achieved in the past 5 yr in the field of stem cells. However, there is no reliable cell source that could be used for the cell-based therapy of diabetes mellitus, so far. In what concerns reprogramming of MSC into functional insulin-producing phenotypes,

there are still a few resources to be explored. Once adequate mechanisms for cell-based therapy are established and tools are successfully applied, it will be possible to obtain cells capable of regenerating or replacing the missing pancreatic β-cells.

It is quite clear that the cure of diabetes will require a combination of distinct strategies. Cell-based therapies may recover endogenous insulin production; yet, modulation of the autoimmune disease behind T1D is essential to achieve an effective and long-lasting therapeutic intervention. Also, β-cell proliferating agents, such as incretin analogs and islet neogenesis-associated protein, might be of benefit when used simultaneously to cell-based therapies, more so on advanced stages of β-cell destruction. Hence, to achieve the goal of permanent insulin independence, work on distinct but complementary research lines has still to go further.

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