Cytotherapy, 2011; 13: 802–813

informa
healthcare

PDX1- and NGN3-mediated *in vitro* reprogramming of human bone marrow-derived mesenchymal stromal cells into pancreatic endocrine lineages

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Abstract

Background aims. Reprogramming of multipotent adult bone marrow (BM)-derived mesenchymal stromal/stem cells (MSC) (BM-MSC) represents one of several strategies for cell-based therapy of diabetes. However, reprogramming primary BM-MSC into pancreatic endocrine lineages has not yet been consistently demonstrated. *Methods*. To unravel the role and interaction of key factors governing this process, we used well-characterized telomerase-immortalized human MSC (hMSC-TERT). Pancreatic endocrine differentiation in hMSC-TERT was induced by two major *in vitro* strategies: (i) endocrine-promoting culture conditions and (ii) ectopic expression of two master regulatory genes of the endocrine lineage, human neurogenin 3 (NGN3) and human pancreatic duodenal homeobox 1 (PDX1). *Results*. Both approaches triggered pancreatic endocrine gene expression, notably insulin, glucose-transporter 2 and somatostatin. Transgenic overexpression of NGN3 and/or PDX1 proteins not only induced direct target genes, such as NEUROD1 and insulin, and but also triggered parts of the gene expression cascade that is involved in pancreatic endocrine differentiation. Notably, ectopic NGN3 alone was sufficient to initiate the expression of specific beta-cell lineage-associated genes, most importantly PDX1 and insulin. This was demonstrated both transcriptionally by mRNA expression and reporter gene analyzes and at a protein level by Western blotting. Such reprogramming of hMSC-TERT cells induced glucose-insensitive insulin biosynthesis and secretion. *Conclusions*. Our results indicate that establishment of glucose-dependent insulin secretion in partially reprogrammed human MSC may depend on additional maturation factors. Moreover, hMSC-TERT provides a suitable cell model for investigating further the molecular mechanisms of reprogramming and maturation of adult MSC towards pancreatic endocrine lineages.

Key Words: *cell model, mesenchymal stromal cells, pancreatic endocrine lineages, reprogramming, telomerase-immortalized human bone marrow-derived mesenchymal stromal cell line (hMSC-TERT)*

Introduction

Loss of pancreatic beta cells is a hallmark of both type 1 and type 2 diabetes (1). Therefore, restoring physiologic beta cell mass by means of islet-cell therapy is considered an alternative clinical option. However, the short period of post-transplant survival is a limitation to this approach (2). Donor material is also scarce and, thus far, no additional sources of human beta cells are available. Consequently, the idea of generating beta cells from non-endocrine precursors/stem cells was encouraged by the evidence that pancreatic duct cells (3) and islet-derived stromal cells (4–8) could be induced to produce and secrete insulin *in vitro*.

Multipotent adult bone marrow (BM)-derived mesenchymal stromal/stem cells (MSC) (BM-MSC) have also been considered an important candidate population for the generation of beta cells (9). MSC were first identified within the BM stromal cell compartment (10). Thereafter, similar populations have also been found in tissues such as fat, trabecular bone, dermis, umbilical cord blood and placenta (11,12). MSC are a heterogeneous mixture of fibroblast-like cells of variable commitment. These cells can give rise to mesenchymal offspring for osteogenic, chondrogenic and adipogenic differentiation and potentially, despite being a controversial hypothesis, other non-mesoderm cells such as neuronal cells and hepatocytes (13,14). Attempts to force differentiation towards other lineages, such as myoblasts and even pancreatic endocrine cells, may require proper selection of subpopulations or reprogramming

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(Received 12 October 2010; accepted 4 March 2011)

ISSN 1465-3249 print/ISSN 1477-2566 online
DOI: 10.3109/14653249.2011.571248

procedures (9,15,16). Furthermore, genetic manipulation of adult human MSC has suggested, in a similar way to human fetal liver cells (17,18), that adult human MSC can be directed along the pancreatic beta cell lineage after ectopic expression of the PDX1 gene (19).

Pancreatic endocrine differentiation, during development is determined by sequential expression of specific transcription factors (20). Of these, pancreatic duodenal homeobox 1 (PDX1) is required to initiate pancreatic organogenesis and to maintain mature beta cell function (21,22). Neurogenin 3 (NGN3) transcription factor is, in turn, the key switch to pancreatic endocrine differentiation (23,24). For this reason, ectopic expression of PDX1 and NGN3 has been utilized to induce insulin production, namely in human embryonic stem cells and pancreatic ductal cells *in vitro* (25), as well as in mouse liver cells *in vivo* (26). Likewise, overexpression of PDX1 in heterogeneous populations of primary BM-MSC has resulted in insulin-producing phenotypes with variable efficiency (19). BM-MSC in culture may lose their capacity to differentiate towards distinct lineages, possibly including endocrine phenotypes, which, as we have demonstrated earlier, can be overcome by telomerase-immortalization (27).

Therefore, as a proof of principle, reprogramming human BM-MSC towards endocrine pancreatic lineages has been demonstrated. However, current reprogramming efforts so far have demonstrated low reproducibility. This may result from the great heterogeneity of BM-MSC populations employed, suggesting that a well-characterized cell model may overcome the limitations of primary MSC and allow study of the molecular mechanisms involved in endocrine pancreatic differentiation pathways.

We have previously shown that BM-MSC are similar to islet-derived stromal cells, which in addition express genes compatible with the process of mesenchymal epithelial or epithelial mesenchymal transition (MET/EMT) (28). Such cells are putatively capable of completing MET and developing into insulin-producing cell types.

In this study, we exploited the potential of a telomerase-immortalized human BM- MSC line (hMSC-TERT) (27,29) to be reprogrammed into endocrine pancreatic lineages. Using permissive culture conditions and forced expression of key transcription factors, we gained insight into molecular pathways activated during endocrine pancreatic differentiation in human MSC.

Methods

Unless otherwise noted, reagents were purchased from Invitrogen GmbH (Karlsruhe, Germany).

Cell culture

hMSC-TERT, as obtained from Moustapha Kassem (Odense, Denmark), were generated by retroviral transduction of BM-MSC from a 33-year-old healthy male donor and were routinely cultured on Earl's Minimum Essential Medium (MEM) with 10% fetal calf serum (FCS), 100 U/mL penicillin (P) and 100 μg/mL streptomycin (S) as described previously (29). Human pancreatic islets were isolated at the Giessen Islet Isolation and Transplantation Centre (Gieesen, Germany) from human 'research' pancreases obtained from brain-dead multi-organ donors after legal consent and approval from the local ethics committee, according to a modified semi-automated digestion-filtration method (30,31). Prior to experiments, islets were cultured for 1–5 days in RPMI-1640 (without glucose) supplemented with 5.6 mM glucose, 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM N_2-hydroxyehtylpiper-azine-N_2-ehtanesulfonic acid (HEPES) buffer, 1 mM sodium pyruvate, 100 U/mL P and 100 μg/mL S.

Human (h) BM-MSC were harvested from the BM of femoral heads from five otherwise healthy patients ($n = 5$) undergoing total hip arthroplasty. Informed consent was obtained from each patient and experiments were performed upon approval of the local ethics committee. Whole BM cells were initially seeded at a density of $6.6 \times 10^5/cm^2$ to obtain hBM-MSC by plastic adherence. After 2–3 days of cultivation, non-adherent cells were removed and adherent cells were washed twice with phosphate-buffered saline (PBS). Cells were cultivated and expanded in Dulbecco's modified Eagle medium (DMEM)/Ham's F12 (1:1) medium supplemented with 10% FCS, 1 U/mL P, 100 μg/mL S and 50 μg/mL L-ascorbic acid 2-phosphate. hBM-MSC at passage 2–3 were used for experiments.

Endocrine differentiation of hMSC-TERT was induced by two approaches: (i) specific medium and (ii) ectopic overexpression of human NGN3 and/or human PDX1 proteins. For approach (i), 2.5×10^5 cells/well were seeded in six-well plates and cultured for 8–9 days in a permissive endocrine differentiation medium. This consisted of serum-free DMEM/F12 containing 17.5 mM glucose, 10 mM nicotinamide, 2 nM activin-A, 10 nM exendin-4, 100 pM hepatocyte growth factor (Sigma-Aldrich GmbH, Schnelldorf, Germany), 1% bovine serum albumin (BSA), 2 mM-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (32,33), called 'cocktail medium'. For approach (ii), we generated transgenic hMSC-TERT cell lines with stable overexpression of NGN3 and/or PDX1 genes. After transfection, cells were kept in standard medium (MEM with 10% FCS and antibiotics) complemented with a selection of antibiotics as described below. Transformed African