Differentiation of nestin-positive cells derived from bone marrow into pancreatic endocrine and ductal cells *in vitro*

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Abstract

Promising results of pancreatic islet transplantation to treat type 1 diabetes mellitus, combined with severe shortage of donor pancreata, have spurred efforts to generate pancreatic islet-like cells and insulin-producing β -cells from various progenitor populations. In this study, we show for the first time that multipotent nestin-positive stem cells selected from rat bone marrow can be differentiated into pancreatic ductal and insulin-producing β -cells *in vitro*. We report an effective multistep protocol in a serum-free system, which could efficiently induce β -cell differentiation from multipotent nestin-positive bone marrow stem cells. To enhance the induction and differentiation toward pancreatic lineage we used trichostatin A, an important regulator of chromatin remodeling, and 5-aza 2' deoxycytidine, an inhibitor of DNA

methylase. All-*trans* retinoic acid was then utilized to promote pancreatic differentiation. We sequentially induced important transcription factor genes, such as *Pdx1*, *Ngn3*, and *Pax6*, following the *in vivo* development timeline of the pancreas in rats. Furthermore, in the final stage with the presence of nicotinamide, the induced cells expressed islet and ductal specific markers. The differentiated cells not only expressed insulin and glucose transporter 2, but also displayed a glucose-responsive secretion of the hormone. Our results delineate a new model system to study islet neogenesis and possible pharmaceutical targets. Nestin-positive bone marrow stem cells may be therapeutically relevant for β -cell replacement in type 1 diabetes.

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Introduction

Understanding the mechanisms of β -cell homeostasis and regenerative repair is crucial not only to provide new insights on diabetes mellitus pathogenesis, but also to address potential therapeutic application. Islet cell transplantation may be a promising approach for cell replacement in type 1 diabetes. However, islet availability for allogeneic transplantation is limited (Lakey *et al.* 2006, Shapiro *et al.* 2006). In addition, understanding the origin of new β -cells in adults may help devise ways of slowing down progression of type 2 diabetes.

The mechanisms by which β -cell mass is maintained in adulthood are not yet known and the origin of new β -cells in adults is the focus of intensive research. The literature is replete with studies trying to characterize a putative pancreatic progenitor cell in and outside the pancreas that could explain β -cell neogenesis in adult life. Should such a cell be identified, it could be used as a potential source of cell therapy. It has been demonstrated that replacement of β -cells in the post-natal period precedes both the proliferation of existing mature β -cells (Dor *et al.* 2004), and the neogenesis of new β -cells from progenitor epithelial cells. It has been proposed that these adult pancreatic progenitor cells reside in

the epithelium of the pancreatic duct (Bouwens & Kloppel 1996, Bonner-Weir 2000, 2001), inside islets (Zulewski *et al.* 2001) and in the bone marrow (Ianus *et al.* 2003).

Nestin is an intermediate filament protein known as a marker of neuroepithelial stem cells, because it is expressed transiently in early developmental stages as well as in the process of tissue regeneration in various organs (Lendahl *et al.* 1990, Morshead *et al.* 1994, Matsuoka *et al.* 2002). Immunohistochemical studies showed the presence of nestin-positive cells within the islet, the acinar and the ductal compartment of the prenatal and newborn pancreas (Hunziker & Stein 2000, Zulewski *et al.* 2001, Kim *et al.* 2004, Yashpal *et al.* 2004). The developmental progression and the change in nestin immunoreactivity throughout the fetal pancreas to postnatal transition suggest that nestin-positive cells are probably a population of progenitor cells within the pancreas (Yashpal *et al.* 2004). Moreover, Zulewski *et al.* (2001) described the existence of a distinct population of cells within islets and in a focal region of pancreatic ducts and exocrine pancreas expressing nestin that can proliferate and differentiate into pancreatic, exocrine, ductal and endocrine cells in culture. A recent publication showed that suppression of nestin expression in embryonic stem cells by gene silencing

reduced endodermal and pancreatic transcription factor expression (Kim *et al.* 2010). During embryonic development, neural and islet cells express a subset of markers in common. Developing islet cells express several neuronal-specific markers, such as synaptophysins, nerve-specific enolase (Alpert *et al.* 1988), and transcription factor genes such as *Isl-1*, *Pax6*, *Pax4*, $\beta 2/\text{NeuroD}$, and *IDX1* (Reynolds & Weiss 1996, Madsen *et al.* 1997, Sander & German 1997).

Therefore, these data suggest that nestin could be a common marker of a precursor stem cell for both neuronal and islet cell types. We previously demonstrated that a subpopulation of nestin-expressing cells, isolated from bone marrow is able to generate cellular spheres similar to neurospheres derived from brain neural stem cells. These cells from bone marrow could differentiate into all three neural phenotypes (neurons, astrocytes, and oligodendrocytes) *in vitro* and *in vivo* (Kabos *et al.* 2002, Zeng *et al.* 2007). Recent studies showed that bone marrow-derived stem cells could reverse the hyperglycemic phenotype in a diabetic animal model but the mechanism behind the rescue or regeneration of pancreatic islets is still debated (Hasegawa *et al.* 2007, Xu *et al.* 2007, Gao *et al.* 2008, Zhao *et al.* 2008). Based on these we hypothesized that nestin-positive cells from bone marrow (bone marrow-derived stem cells, n-BMSCs) could be differentiated to pancreatic cell phenotypes by serial epigenetic manipulations.

We reported a newly developed and effective method that could successfully induce directly n-BMSC to differentiate into pancreatic cell lineage. In the first step (chromatin remodeling) we used an inhibitor of DNA methylase, and 5-aza 2' deoxycytidine (5-AZA), which has been shown to be important in differentiation process, including pancreatic differentiation (Lefebvre *et al.* 2009), and a histone deacetylases (HDACs) inhibitor, trichostatin A (TSA), to loosen chromatin structure and permit differentiation through epigenetic manipulations (Chang *et al.* 2004). In the second step, retinoic acid (RA) was used to promote pancreatic differentiation, consistent with the previous observation that RA is required for early pancreas development in human and mice (Ostrom *et al.* 2008). In the last step, we used nicotinamide, which has been reported to induce differentiation/maturation of precursor/stem cells toward a β -cell phenotype and enhance insulin biosynthesis (Ye *et al.* 2006).

This system can provide a novel source for generating β -cells and can be utilized as a model to study the mechanism of islet neogenesis, which will facilitate identifying pharmaceutical targets to treat insulin-dependent diabetes.

Materials and Methods

Cell isolation and culture

Adult Fisher rats were purchased from Charles River Laboratories (Wilmington, MA, USA). They were killed by CO₂ asphyxiation according to an approved protocol by the

Institutional Animal Care and Use Committee at Cedars Sinai Medical Center. Whole bone marrow was harvested from the femurs as previously published (Talmor *et al.* 1998). Briefly, the femurs were isolated from the muscle tissues and both ends of the bones were cut. The marrow was flushed and the tissue was passed through a mesh to remove small pieces of debris. After washing, nestin-positive spheres were obtained as described previously by our group (Kabos *et al.* 2002). Cells were plated at a density of 1×10^6 cells/well in poly-D lysine-coated 24-well plates (BD Biosciences, San Jose, CA, USA) in serum-free DMEM/F12 medium (Invitrogen), supplemented with 20% B27 (Invitrogen), 20 ng/ml of fibroblast growth factor (bFGF, Peprotech, Rocky Hill, NJ, USA), 20 ng/ml of epidermal growth factor (EGF, Peprotech), penicillin (100 U/ml) and streptomycin (100 μ g/ml). Medium was changed every 2 days. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. Portions of cells from sphere forming were dissociated to single cells, frozen down (10% dimethylsulfoxide and 40% fetal bovine serum) by cryotank in -80 °C for overnight and put into the liquid nitrogen (LN2) tank for maintenance of cell populations.

Figure 1 Differentiation steps of multipotent nestin-positive stem cell isolated from rat bone marrow (n-BMSC). Multipotent rat bone marrow stem cells formed neurospheres *in vitro* (A) (magnification $\times 10$) and stained positive for nestin (B) (magnification $\times 20$). (C) Schematic representation of multistep differentiation protocol of n-BMSC to pancreatic lineage (endocrine and ductal phenotype): chromatin remodeling with 5-AZA and TSA (step 1), induction with ITS and RA (step 2), differentiation (step 3), and maturation with nicotinamide (step 4). Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-10-0344>.

For control experiment rat bone marrow mesenchymal stem cells (MSCs-BM) were cultured on α -MEN medium (Invitrogen) supplemented with 10% FCS (Invitrogen). Briefly, the cells collected from bone marrow (as described earlier) were plated into tissue culture-treated dishes of 100 mm in diameter at 37 °C in a humidified atmosphere containing 5% CO₂. After 24 h, floating cells were washed out and only adherent cells were maintained in the culture with medium changed every 3 days. Cells which reached 80–90% confluency in 100 mm dish were passaged to 1:4 and cultured in the same medium.

Cell culture and differentiation

We have previously described the successful selection, from rat whole bone marrow, of multipotent stem cells expressing nestin (n-BMSC) and forming a spheroid structure with phenotypic characteristics and a genetic signature typical of neurospheres (Kabos *et al.* 2002).

We established a complex differentiation protocol comprised of four basic steps (Fig. 1). Control experiments were conducted in parallel using rat MSCs-BM. In the first step (chromatin remodeling), nestin-positive spheres were plated at a concentration of about 100 spheres/ml in each well of 6-well culture plate (Corning, Corning, NY, USA), in a basal medium containing KO-DMEM and main components: 0.1 mM β -mercaptoethanol, 2 nM L-glutamine, 1% non-essential amino acid, 0.2% BSA, N2 supplement, B27 supplement (all from Invitrogen), 2 μ g/ml heparin, 20 ng/ml bFGF, and 20 ng/ml EGF. The cells were treated with 1 μ M 5-AZA (Sigma) for 24 h. After 24 h the medium was changed and cells were treated with 100 nM TSA (Sigma) for 24 h. In the second step (induction), the basal medium was changed

with induction medium containing DMEM with low glucose (1 g/l; Invitrogen), 1 $\times$ insulin–transferin–selenium (ITS; Invitrogen), 2 μ M all-*trans* RA (Sigma), and main components. The cells were maintained in induction medium for about 7 days. In the third step (differentiation), the cells were seeded into 6-well plates coated with poly-L-ornithine (15 μ g/ml; Sigma) at a concentration of 2–5 $\times$ 10⁵ cells/well in differentiation medium containing DMEM with high glucose (Invitrogen) and main components for 7 days. In the last step (maturation) the medium was modified from differentiation medium, adding 10 mM nicotinamide (Sigma) without supplement of bFGF and EGF for 7 days. For each step the medium was changed every 2 days.

Quantitative real-time PCR

Cells were harvested at different steps and total RNA was extracted using RNeasy Minikit (Qiagen), according to the manufacturer's instruction. cDNA was prepared using Superscript reverse transcriptase (Invitrogen). cDNA samples derived from 50 ng of total RNA was analyzed by quantitative reverse transcription-PCR (qRT-PCR) using SYBR green dye with QuantiTect SYBR Green RT-PCR kit (Qiagen). Sequences of PCR primers used are listed in Table 1. PCR was performed with cycles at 95 °C for 15 s, 56–60 °C for 10 s (Table 1) and 72 °C for 20 s. Reactions (40 cycles) were carried out with iCycler PCR machine (Bio-Rad) and data analysis was performed with QPCR software (Applied Biosystems, Foster City, CA, USA). Each experiment was performed at least three times. Relative quantitative analysis was performed following 2^{− $\Delta\Delta C_T$} . The expression of each gene was normalized to β -actin gene expression.

Table 1 PCR primer sets

	Primer sequences	Annealing T (°C)/time (s)	References
Nestin	F: 5'-gcggggcggtgcgtgactac-3' R: 5'-aggcaagggggaagagaaggatgt-3'	58/10	Zulewski <i>et al.</i> (2001)
Pdx1	F: 5'-atcactggagcaggaagt-3' R: 5'-gctactacgtttcttatct-3'	56/10	Zulewski <i>et al.</i> (2001)
Ngn3	F: 5'-ccgctggagtgacctctaa-3' R: 5'-ggtggaattggaactgagcactt-3'	60/10	Calderari <i>et al.</i> (2007)
CK19	F: 5'-acagccagctactcaagacc-3' R: 5'-ctgtgtcagcacgcacgtta-3'	56/10	Zulewski <i>et al.</i> (2001)
Insulin	F: 5'-tcttctacacacccatgtccc-3' R: 5'-cacctagtcacgacgtgg-3'	55/10	Jonas <i>et al.</i> (1999)/J00747-8
Glucagon	F: 5'-acctagactccgcccgtg-3' R: 5'-cttgaacccgcgtctgta-3'	55/10	Jonas <i>et al.</i> (1999)/K02808
PP	F: 5'-cgcatactactgcctctccc-3' R: 5'-cagcagcgcaggcatcaaa-3'	60/10	Choi <i>et al.</i> (2005)
Som	F: 5'-ctgcatctctctggctttgg-3' R: 5'-tgcagccagctttgcgtcc-3'	55/10	Choi <i>et al.</i> (2005)
Glut2	F: 5'-ggatctgctcacatagtcac-3' R: 5'-ccaagtaggatgtgccagta-3'	60/10	Choi <i>et al.</i> (2005)
β -actin	F: 5'-acctgacagactacacctatg-3' R: 5'-atcgctactctgcttgctga-3'	58/10	Choi <i>et al.</i> (2005)

PP, pancreatic polypeptide; Som, somatostatin; Glut2, glucose transporter 2.

Immunocytochemistry

Spheroids were fixed in 4% paraformaldehyde in phosphate buffer for 10 min. Blocking was carried out for 30 min using 10% FCS diluted in PBS at room temperature. The cells were then incubated with primary antibodies in solution of 3% of FCS in PBS for an hour at room temperature. Antibody dilutions were as follows: mouse monoclonal anti-nestin (MAB353) (1:100; Millipore Corporate, Bellerica, MA, USA), goat anti-PDX1, rabbit anti-PAX6, rabbit anti-CK19, rabbit anti-insulin, goat anti-glucagon, and rabbit anti-amylase (All 1:50, from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Afterwards, the cells were washed with PBS for 5 min for three times. Secondary antibodies were FITC or Texas red conjugated anti-mouse, anti-goat and anti-rabbit (Vector Laboratories, Inc., Burlingame, CA, USA) and were diluted 1:500 in PBS containing 3% of FCS. Cells were incubated in this solution for 45 min at room temperature, followed by washes in PBS for 5 min for three times. Matched exposure of control sample was stained using non-immune calf serum in place of the primary antibodies. Nuclear DNA was stained

with 4',6-diamidino-2-phenylindole (Vector Lab). Images were captured by digital camera connected with fluorescent microscope (Model Upright Zeiss, Jena, Germany). Scanning confocal images were obtained by a confocal microscope (Leica Microsystems SP5, Mannheim, Germany).

Dithizone staining

Dithizone (DTZ, Sigma), which stains zinc-containing cells bright red, was used to quickly assess the presence of insulin-producing cells. The staining protocol was followed from the study by Shiroy *et al.* (2002). DTZ stock solution was prepared by solving 50 mg of DTZ in 5 ml of dimethyl sulfoxide (Sigma), sterile-filtering through a 0.22 µm nylon filter, and stored at -20 °C. The working solution was prepared (pH 7.8) by diluting the stock solution 1:100 in culture medium. For each dish, 2 ml of DTZ solution was added and incubated for 30 min at 37 °C. After washing the cells three times in PBS, the differentiated islet-like clusters were examined under phase contrast inverse microscope.

Figure 2 Expression of nestin and pancreatic transcription factors during *in vitro* n-BMSC differentiation. (A–D) Immunofluorescence staining for nestin was positive (A) in the beginning and negative in the following differentiation step. (E–H) Immunofluorescence for PDX1 was negative in the beginning (A) and positive in the three following steps. (I–N) Immunofluorescence staining for PAX6 was negative in the beginning (A); positive in only few cells by step 2 (L), and in many cells by step 3 (M); negative again in the last step (N). Nuclei were counterstained with DAPI. Confocal microscopy, original magnification $\times 63$ (A, F, G, and H); Fluorescent microscopy $\times 40$ (M and N).

Figure 3 Quantitative reverse transcription-PCR analysis of n-BMSC for *Pdx1* and *Ngn3* (A): *Pdx1* and *Ngn3* genes were not expressed in the beginning of pancreatic differentiation *in vitro* but were induced during step 2 of the differentiation protocol. ΔC_T presents the difference of the cycle threshold between the assayed gene and the normalizing gene. (B) Variation of gene expression during *in vitro* differentiation. Values are fold change $\pm$ s.d. ($n=3$). * $P<0.05$; ** $P<0.01$.

Insulin assay

For each determination, about 100 spheroids of similar size were randomly handpicked at stage 4 (3 weeks after starting the pancreatic differentiation) and incubated in DMEM with low glucose (1 g/l) without serum overnight. For insulin secretion assays spheroids were preincubated for 1 h in Krebs–Ringer/bicarbonate buffer (KRB: 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 1.1 mM MgCl_2 , and 25 mM NaHCO_3) at 37 °C or on ice (for control experiments) in 5% CO_2 atmosphere, followed by incubation for an additional hour in KRB containing 0.5 mM 1-isobutyl-3-methylxanthine and glucose at different concentrations (5, 10, and 20 mM). The buffer was collected and frozen at -70 °C until assay for insulin content. The amount of insulin released was determined by ultrasensitive rat insulin ELISA (Linco Research, Billerica, MA, USA) according to the manufacturer's protocol. Values showed the mean of three replicates $\pm$ s.d.

Statistical analysis

Results are shown as mean $\pm$ s.d. Student *t*-test was used to compare between two groups. One-way ANOVA was used to compare among three groups. In all statistical analyses, a *P* value of <0.05 was judged statistically significant.

Results

To drive differentiation of bone marrow stem cells expressing nestin towards pancreatic lineages, we developed a culture procedure comprising four steps (Fig. 1). In the first step the cells were exposed to 5-AZA for 24 h and TSA for another 24 h. The dose of 5-AZA used in our study has been

successfully used by Lefebvre *et al.* (2009) to induce NGN3 expression and endocrine differentiation into the PANC-1 human ductal cell line. We determined the optimal concentrations of TSA by testing cell survival, growth and death with increasing doses from 10 nM to 1 mM. Concentrations of more than 100 nM for TSA showed an increased cell death and reduced differentiation capability (data not shown). We rationalized that chromatin remodeling is the first step to induce nestin-positive cells because of putative cell lineage differences and specification, and hypothesized that the increased 'susceptibility' of chromatin by serial epigenetic modifications will enhance the induction and further direct differentiation toward the pancreatic phenotype (Fig. 1). After exposure to TSA, the cells were cultured for 7 days in a serum-free medium containing ITS and RA (Fig. 1C, induction). The concentration of RA adopted in our study was previously optimized in embryonic stem cell differentiation model (Cai *et al.* 2009). Recently, the role of RA in pancreatic development and early endocrine lineage determination has been shown (Ostrom *et al.* 2008). In addition, RA has been used for both mouse and human embryonic stem cells to induce differentiation into pancreatic cell lineage (Micallef *et al.* 2005, Johannesson *et al.* 2009). At this time point we were able to highly induce the expression of a pancreatic master gene (pancreas duodenum homeobox-1, *PDX1*), together with other genes important in the early pancreatic development (*PAX6* and *NGN3*). Many cells were positive for *PDX1* as assayed by immunohistochemistry (Fig. 2F). Subsequently, the *PDX1*-positive cell population dramatically decreased after 7 days during the third step (differentiation; Fig. 2G) but increased again and became prominent in the fourth step (maturation; Fig. 2H). This fluctuation of *PDX1*-positive cell population by immunohistochemistry also appeared in the qRT-PCR analysis in sequential manner (Fig. 3B). *Pdx1* gene expression was initiated within 7 days during the second step culture

Table 2 Expression of pancreatic transcription factors and islet hormone transcripts at different time points during *in vitro* differentiation

	Time 0	Step 2 (day 9)	Step 3 (day 16)	Step 4 (day 23)
Nestin	26 $\pm$ 1	–	–	–
<i>Pdx1</i>	–	29 $\pm$ 0.5	32 $\pm$ 1	27 $\pm$ 1
<i>Ngn3</i>	–	28 $\pm$ 0.2	27 $\pm$ 0.2	26 $\pm$ 0.1
Insulin	–	–	27 $\pm$ 0.2	23 $\pm$ 1
Glucagon	–	–	29 $\pm$ 0.1	26 $\pm$ 0.2
<i>Glut2</i>	–	–	29 $\pm$ 0.1	27 $\pm$ 0.5
<i>Ck19</i>	–	–	25 $\pm$ 0.1	23 $\pm$ 0.1
Amylase	–	–	–	–
Somatostatin	–	–	–	–
PP	–	–	–	–

RNA from n-BMSC was extracted at different time points during *in vitro* differentiation and analyzed by quantitative reverse transcription-PCR for transcripts encoding pancreatic transcription factors and the four islet hormones. Values represent cycle threshold $\pm$ s.d. PP, pancreatic polypeptide.

(induction), then downregulated several fold but was detectable ~ 2 weeks later during the third step, and upregulated again 3 weeks later during the maturation step. *Ngn3* transcript was induced at day 7 and progressively increased two- to fivefold at day 14 (step 3) and day 21 (step 4) respectively (Fig. 3). Only a few cells were positive for PAX6 at day 9, as confirmed by immunohistochemistry (Fig. 2) and the number of positive cells strongly increased during step 3, but was not detectable at step 4. Surprisingly, these results were consistent with the physiological expression of *PDX1*, *PAX6*, and *NGN3* genes during *in vivo* pancreas development. In contrast, nestin expression was high in the beginning of the differentiation and dramatically decreased to an undetectable level during the second stage of conditioning. This was confirmed by both immunohistochemistry and (Fig. 2A–D) and qRT-PCR (Table 2). Morphologically, the cells stayed aggregated in spheroid structure.

In the third stage of conditioning we used poly-L-ornithine-coated dishes with a medium containing high glucose with both bFGF and EGF. In such an environment, the cells underwent further differentiation into a pancreatic phenotype. At about 2 weeks of the differentiation process, corresponding to the maximized expression of PAX6, increased expression of NGN3 and downregulation of PDX1, endocrine markers for a more mature phenotype started to appear as confirmed by qRT-PCR: insulin, glucagon, and glucose transporter 2 (*Glut2*; Table 2). The expression of *Ck19*, a ductal marker, was also induced. At this stage immunohistochemistry was negative for the same markers (Fig. 4).

In the last step, we tried to further push the differentiation toward a more mature phenotype. For this reason we used nicotinamide for about 7 days. At day 21 from the beginning of the *in vitro* differentiation, we observed a mixed population of cells expressing endocrine and ductal phenotypes. Immunofluorescent staining was markedly positive for insulin and CK19 (Fig. 4). These cells also positively stained with the zinc chelator, DTZ, indicating the presence of intracellular proinsulin, as has been previously observed for 'pseudoislets' in culture (Kuo *et al.* 1992). Only a small number of cells were positive for glucagon with immunofluorescent staining (data not shown). Insulin, CK19, and glucagon transcripts were also consistently upregulated (seven- to eightfold, five- to sixfold and two- to threefold, respectively; Fig. 5A and B). No amylase was detected on immunohistochemistry (data not shown) and RNA levels (Table 2), indicating the absence of a pancreatic exocrine phenotype. In our culture condition we also failed to detect somatostatin and pancreatic polypeptide-positive cells (Table 2).

To evaluate the functionality of differentiated cells, we tested for glucose-induced insulin secretion *in vitro*. At the end of the last differentiation stage, the cell aggregate (spheroids) not only produced insulin, but also displayed a glucose-responsive secretion of the hormone. Insulin secretion was dose-dependent after incubation for 1 h with glucose (5, 10, and 20 mM; Fig. 5C). Spheroids incubated on ice under

Figure 4 Differentiation of n-BMSC in a mature pancreatic phenotype. Immunofluorescence staining for CK19 (A and B) and for insulin (C and D) was negative until the third differentiation step and became positive in the last step (B and D). Dithizone staining for proinsulin (E and F) was also positive in the last differentiation step. Nuclei were counterstained with DAPI. Images of insulin staining visualized under confocal microscope (D–G) with detail of single-cell sectioning (H) showing the insulin granular cytoplasmatic pattern. Confocal microscopy, original magnification $\times 63$ (B, D, G, and H); phase contrast microscopy, original magnification $\times 20$ (E and F).

the same condition did not show glucose-responsive insulin secretion. The average insulin secretion on ice with glucose 5, 10, and 20 mM was 0.85 ± 0.32 , 1.5 ± 0.41 , and 1.8 ± 0.5 ng/100 spheroids per hour respectively. This is consistent with inhibition of glucose-induced insulin release by cooling (Atwater *et al.* 1984). These findings demonstrate *de novo* synthesis and processing of insulin and physiologically regulated secretion. Moreover the cells expressed a functional element, *Glut2* (Fig. 5A and B) that allows the rapid entry of glucose into the cells. This confirmed the capability of our differentiated cells to respond to glucose stimulation.

Figure 5 Quantitative reverse transcription-PCR analysis of n-BMSC. (A) Gene not expressed in the beginning of pancreatic differentiation *in vitro* and induced during step 3 of the differentiation protocol. ΔC_T represents the difference between the cycle threshold of the assayed gene and that of the normalizing gene. (B) Variation of gene expression during *in vitro* differentiation. (C) Insulin production in response to glucose concentration: n-BMSCs were incubated in a buffer containing the indicating concentration of glucose. Values are fold change $\pm$ S.D. ($n=3$). ** $P<0.01$; *** $P<0.005$; **** $P<0.0001$.

Discussion

Promising results of pancreatic islet transplantation to treat diabetes mellitus, combined with severe shortage of donor pancreata, have spurred efforts to generate insulin-secreting β -cells from various progenitor populations. In this study, we report a novel culture system in order to obtain insulin-producing cells derived from nestin-positive bone marrow cells in rats (n-BMSC). Our procedure successfully induced n-BMSC to express lineage-specific transcription factor, in particular *Pdx1*, which has been shown to drive pancreatic differentiation.

We pretreated the cells with 5-AZA to inhibit DNA methylation, which has been recognized in important differentiation processes, such as osteoblast (Vaes *et al.* 2009), cardiomyocyte (Tomita *et al.* 1999) and more recently pancreatic endocrine differentiation (Lefebvre *et al.* 2009).

We sequentially treated the cells with TSA based on the hypothesis that chromatin remodeling will enhance the induction and further differentiation toward pancreatic lineage. Acetylation or deacetylation of histone terminal domains can regulate gene expressions. Histone acetyltransferases and HDACs can loosen or compact chromatin structures and regulate cell proliferation and differentiation in various tissues (Zhang *et al.* 2002, Chang *et al.* 2004). As the acetylation status of nucleosomal histone modulates chromatin structure and epigenetically regulates gene expression, an inhibitor of both classes I and II HDACs such as TSA may be used to loosen the chromatin structure and more easily permit differentiation through epigenetic manipulation. Moreover, a recent study from Haumaitre *et al.* (2008) showed that HDAC inhibitors can modify the timing and the determination of pancreatic cell fate with inhibition of exocrine and enhancement of ductal and endocrine differentiation. In our experiment, after exposure to 5-AZA and TSA, the cells lost nestin expression and after being cultured in a medium containing ITS and RA, they also turned on expression of mRNA encoding transcription factors such as *Pdx1*, *NgN3* and *Pax6*, well known to play important roles in the developmental program leading to the formation of pancreatic islets (Jensen 2004). Moreover, the expression of these transcription factors followed the timing consistent with the normal mammalian pancreatic development. PDX1 was expressed in the second step after exposure to RA and ITS medium and subsequently expression of both PAX6 and NGN3 were activated. In the last step, a more mature phenotype of ductal and endocrine pancreatic cells appeared. These cells lost the expression of PAX6, and upregulated PDX1, insulin, glucagon, GLUT2 and CK19.

Another crucial step in our differentiation protocol is the use of RA to induce differentiation of n-BMSC towards a pancreatic phenotype. In our experiment it was extremely important to time the exposure to RA, right after the cell treatment with TSA. RA has been shown to control multiple steps in the motor neuron differentiation in the ventral spinal cord (Novitsch *et al.* 2003). In an elegant experiment, Ostrom *et al.* (2008) showed that RA is present in the developing mouse and human pancreas and is required for pancreas development. Moreover, all-*trans* RA has been widely used to promote generation of PDX1-positive pancreatic progenitor cells from mouse embryonic stem cells (Micallef *et al.* 2005, Shi *et al.* 2005) and to induce pancreatic differentiation in human embryonic stem cells (D'Amour *et al.* 2006, Johannesson *et al.* 2009). In our experiment, consistent with the previous observations, we showed that RA induces expression of PDX1.

In the last step both ductal and endocrine phenotypes were present and this is probably because the activation of *Pdx1* in the second step of our protocol, subsequently activated both the endocrine and the non-endocrine pathways. *Pdx1* is well known for controlling early whole pancreas development.

Our starting hypothesis was that a selected stem cell population from rat whole bone marrow highly expressing nestin could be a potential multipotent precursor stem cell able to differentiate to pancreatic cell lineage. Our hypothesis was based on the evidence that nestin is a neuroepithelial marker transiently expressed in early stages in many tissues, including pancreas, and that nestin-positive cells from pancreas can be differentiated in culture into endocrine, exocrine, and ductal cells (Zulewski *et al.* 2001).

We demonstrated that epigenetic manipulation of n-BMSC, following a specific multistep protocol, can induce expression of transcription factors involved in the early pancreatic and endocrine specification in a step-wise fashion whose timing was consistent with normal pancreas development. Differentiation into a more mature phenotype, including ductal and insulin-producing cells was efficiently induced. It remains to be shown how long these cells remain in this differentiated state both *in vitro* and *in vivo*. In conclusion, we propose a novel cellular system that can address these unmet needs, both for cell therapy and for mechanistic studies and drug discovery in the field of pancreatic islet neogenesis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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