

# Induction of Nestin Early Expression as a Hallmark for Mesenchymal Stem Cells Expression of PDX-1 as a Pre-disposing Factor for Their Conversion into Insulin Producing Cells

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Diabetes constitutes a worldwide epidemic that affects all ethnic groups. Cell therapy is one of the best alternatives of treatment, by providing an effective way to regenerate insulin-producing cells lost during the course of the disease, but many issues remain to be solved. Several groups have been working in the development of a protocol capable of differentiating Mesenchymal Stem Cells (MSCs) into physiologically sound Insulin Producing Cells (IPCs). In order to obtain a simple, fast and direct method, we propose in this manuscript the induction of MSCs to express NESTIN in a short time period (2 h), proceeded by incubation in a low glucose induced medium (24 h) and lastly by incubation in a high glucose medium. Samples from cell cultures incubated in high glucose medium from 12 to 168 h were obtained to detect the expression of INSULIN-1, INSULIN -2, PDX-1 and GLUT-2 genes. Induced cells were exposed to a glucose challenge, in order to assess the production of insulin. This method allowed us to obtain cells expressing PDX-1, which resembles a progenitor insulin-producing cell.

**Keywords:** Mesenchymal stem cells, PDX-1, Insulin producing cells, Nestin

## Introduction

Diabetes mellitus is a chronic disease that has been present along human history and unfortunately, its prevalence in recent decades has reached pandemic proportions. The disease is characterized by a group of metabolic disorders such as chronic hyperglycemia, which is caused in

some instances by an absolute or relative deficiency of insulin, the key hormone in the maintenance of normal glucose levels. Some pathological factors of diabetes are the destruction of beta cells, insulin resistance, insufficient insulin secretion in response to the stimulation of glucose, proinsulin defective synthesis, the inability to carry insulin to a mature stage, and a decrease in the proliferation of beta cells (1-3).

Even though systemic management of diabetes has improved patients quality of life, the disease remains an unsolved health problem of epic proportions. For several years, exogenous insulin for type I and some cases of type II diabetes has been the standard treatment available for patients suffering from this condition (4).

Some alternative therapies have been proposed to this treatment, like the replacement of lost beta cells by transplantation of pancreatic Islets of Langerhans (5-9), but this type of therapy confronts the problem of shortage of donors, and the need for immunosuppressive therapies

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(10). Another alternative is the transplantation of stem cells, as well as the differentiation of stem cells into insulin producing cells (IPCs), which have been developed *in vitro* from several protocols (11-13).

Although successful embryonic cell differentiation into insulin producing cells has been reported (14-16), the use of these cells raises severe objections due to their teratogenic capacity and ethics concerns. An alternative to these issues is the use of adult stem cells, which also have the capacity to differentiate to IPCs *in vitro*.

The transcription factor pancreatic duodenal homeobox 1 (Pdx1) plays a key role in the differentiation of non- $\beta$ -cells into IPCs. Based on that fact, stem cells have been transfected with synthesized PDX-1 mRNA (17-19) and the best results were obtained when Nestin expression - a neurofilament present in pancreatic progenitor cells related with the maturity of IPCs (20-22) preceded the synthesis of the transcription factor.

Regardless of the hopeful results obtained thus far, many of the proposed protocols have raised concerns due to the use of transfection techniques and the propensity of neoplastic conversion of these cells, especially after an *in vitro* long-term culture (23).

The objective of this research is the development a short protocol for *in vitro* differentiation of rat bone marrow stem cells into insulin progenitor cells by induction of Nestin synthesis, followed by the expression of PDX-1, and the capability of insulin production after a glucose challenge.

## Materials and Methods

### Ethics statement

Animal studies were performed after approved by the Animal Ethics Committee from the Medical School at the "Instituto Tecnológico y de Estudios Superiores de Monterrey" (ITESM), Reg. 2009-Re-001. All surgeries performed in the animals were under anesthesia and all efforts were made to minimize animal suffering. Animals were housed five per cage at 20~22°C on a 12/12 h light/dark cycle, with food and water *ad libitum*.

### Mesenchymal Cell Isolation and Culture

MSCs were obtained from adult male Wistar rats, weighing ~220 g. Hank's balanced salt solution (Gibco, Grand Island, NY) was used to extrude the bone marrow from femurs and the suspension was filtered using a 70  $\mu$ m pore size cell strainer (BD Falcon, Bedford, MA). After centrifugation, cells were resuspended in Dulbecco's modified Eagle's medium (DMEM-F12; Gibco) containing

20% fetal bovine serum (FBS; Gibco) and 1% antibiotics (streptomycin-penicillin) (Gibco). This medium was used as control medium (CM). Cells were seeded in 100 mm culture dishes (Corning Inc., New York, NY) at 37°C and 5% CO<sub>2</sub> in a humid chamber for 24 h. To remove non-adherent cells, cultures were washed with phosphate-buffered saline (PBS; pH 7.4) and the culture medium was replaced with DMEM-F12 containing 10% FBS and 1% antibiotics. *Neuro-Induction Medium (NIM)*: NIM consisted of CM plus 0.1  $\mu$ M Retinoic acid (RA; Sigma-Aldrich, St. Louis, MO), 1 mM  $\beta$ -mercaptoethanol (Sigma-Aldrich), 2 mM glutamine (Invitrogen, Grand Island, NY), 10 ng/ml fibroblast growth factor 2 (FGF2; Sigma-Aldrich), 0.2% dimethyl sulfoxide (DMSO; Sigma-Aldrich), 40 ng/ml of epithelial growth factor (EGF; Invitrogen) and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich). *Low glucose pre-differentiation medium (LGM)*: LGM consisted in L-DMEM (5.6 mM glucose) (Gibco) containing 20% FBS and 1% antibiotics plus 10 mM Nicotinamide, 0.1  $\mu$ M Retinoic acid, 1 mM  $\beta$ -mercaptoethanol, 4 mM L-glutamine, 0.1% DMSO. *High glucose PDX-1 Induction medium (HGM)*: HGM consisted in (L-DMEM plus 10% FBS, 3.5 g glucose (25 mM glucose) and 1% antibiotics.

### Induction protocol

Samples of  $5 \times 10^4$  cells were seeded on a 24 wells microplate treated with poly-L-lysine (Sigma-Aldrich) and incubated for 2, 4, 6 and 12 h in IM. At the end of each time period, the medium was removed and cells washed with PBS. Total RNA was extracted in order to determine the time of Nestin expression, this time was considered as the "pre-incubation goal" and used for the pre-differentiation protocol. After 24 h of incubation in a low glucose medium (LGM), the medium was removed and replaced by a high glucose medium (HGM). Cultures were incubated for seven days; the medium was change with fresh HGM every three days until reaching seven days of culture. Samples for immunocytochemistry and RT-PCR analysis were collected at a specified time between 12 to 168 h. Additional cells were cultured directly with HGM, without pre-incubation with NIM or LGM and analyzed as experimental cultures. All cultures were maintained at 35°C and 5% CO<sub>2</sub>.

### Immunocytochemistry

Circular glass slides treated with poly-L-lysine (Sigma Chemical Co., St. Louis, Missouri) were placed in 24-well microplates and cells seeded at a density of  $1 \times 10^5$  cells/well. After performing the induction protocol, cells were fixed in 4% paraformaldehyde for 10 min and were

washed three times with PBS (pH 7.4). Cells were permeabilized using 0.3% Triton X-100 in PBS for 5 min. Non-specific antibody reactions were blocked with 5% BSA in PBS for 1 h. Next, cells were incubated overnight at 4°C with primary mouse monoclonal antibodies diluted in PBS containing 1% BSA. The monoclonal mouse antibodies and dilutions used in the experiment were: Anti-nestin mouse monoclonal (10  $\mu$ g/ml; R&D Systems, Minneapolis, MN), Anti-insulin (Sigma), Anti-Glut-2 goat polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA), Anti-PDX-1 goat polyclonal IgG (Santa Cruz Biotechnology), and as secondary antibody was used Anti-mouse IgG antibody conjugated goat Rhodamida (Immunopure<sup>®</sup>) and Anti-mouse IgG antibody conjugated goat FITC (Pierce Biotechnology, Rockford, IL), and goat anti-mouse IgG-FITC (Santa Cruz Biotechnology). Cells were washed three times with PBS and incubated with secondary goat anti-mouse Fc- fluorescein isothiocyanate (1:400; Thermo Fisher Scientific, Pierce Biotechnology, Rockford, IL) for 2 h in the dark. Cells were incubated for 1 min in propidium iodide (0.01 mg/ml; Fluka, Toluca, Mexico) or 4',6-diamidino-2-phenylindole (DAPI; Santa Cruz Biotechnology) to counterstain the nuclei. Cells were analyzed using a fluorescence microscope (Imager Z1; Zeiss, Jena, Germany). Images were obtained using an Axiocam HRm camera system (Zeiss, New York, NY) coupled to the microscope.

### RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from undifferentiated mesenchymal cells and from HGM incubated cells, using a binding silica column kit (GenElute Mammalian Total RNA; Sigma-Aldrich). The amount and quality of total RNA was determined using a GeneQuant pro spectrophotometer (Amersham Biosciences, Cambridge, UK). RT-PCR was performed using a PX2 Thermo thermal cycler (Thermo Fisher Scientific), one-step reactions (Qiagen, Crawley, UK) and the following primer targets: glyceraldehyde-3-phosphate-dehydrogenase (GAPDH; sense, GGTGAAGGTCG-GTGTGA, and antisense, CATGAGCCCTTCCACGA. NESTIN; sense, AACACAGGAGTGGGAAGCTG, and antisense TCTGGCATTGACTGAGCAAC. INSULIN-1; sense, GGGAAACGTGGTTTCTTCTACAC, and antisense GTG-GTGGACTCAGTTGCAGTAG. INSULIN-2; sense, ACC-TTTGTGGTTCTCACTTGGT, and antisense GTAGAGA-GAGCAGATGCTGGTG.

GLUT-2; CTGGGAAGAAGAGACTGAAGGA, and antisense ATACGCTTCTTCCAGCAATGAT. PDX-1; AACCGAGGAGAATAAGAGGAC, and antisense CTTGTGT-

GTGGCGTTTAGGTTA.

### Insulin detection

For insulin detection, cells in HGM culture from each incubation time (12~168 h) were washed five times with Krebs-Ringer buffer and incubated for 2 h with the same buffer to ensure removal of glucose; those cells were stimulated with the addition of HGM and incubated at 35°C and 5% CO<sub>2</sub>, for 60 minutes. The supernatant were analyzed with ELISA kit specific for rat insulin (Alpco Diagnostics, Salem, NH). Absorbance was read in the Synergy HT Microplate reader at 450 nm. The absorbance data standards, controls and samples were recorded with Gen5 software.

### Dithizone staining

Cells in HGM medium cultured for 168 h (St Luis, MO, USA) were incubated at 37°C for 30 min in Dithizone (DTZ) (Sigma-Aldrich). *In vitro* DTZ staining was performed by adding 10  $\mu$ l stock solution to 1 ml culture medium. Stock solution was prepared by dissolving 50 mg DTZ in 5 ml dimethyl sulfoxide [Zhang L, 2005]. After staining, the wells were rinsed three times with Krebs-Ringer buffer and crimson-red-stained clusters were examined with a microscope to determinate the number of DTZ positive cells.

### Statistical analysis

Each experiment was performed in triplicates with three different batches of mesenchymal stem cells each. The data was performed using Minitab software 14 through an analysis of variance (ANOVA).

## Results

### Differentiation of MSC to NESTIN expression

In order to determinate the point of Nestin synthesis induction, cells were incubated in NIM for 2 to 12 h. After this time range was achieved, RNA was obtained. RT-PCR showed an increase of NESTIN expression as early as 2 h of incubation (Fig. 1A). Immunocytochemistry also show nestin distribution inside the cytoplasm at 12 h after induction (Fig. 1B). Considering that 2 h of incubation in NIM was capable to induce nestin synthesis this time of pre-incubation was choosed to continue with the pre-differentiation protocol. Cells incubated with CM did not express NESTIN.

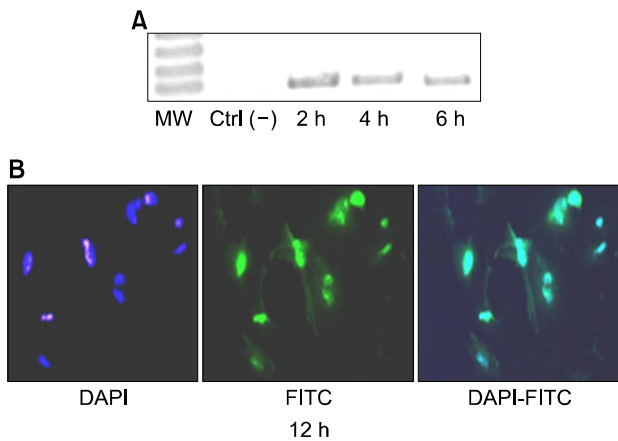
**Differentiation of MSC into IPC. Gene expression by RT-PCR**

mRNA of cells incubated for 30 min with HGM showed positive gene expression for all analyzed genes after 12 h of incubation. The glucose channel GLUT-2 was detected at its highest level after HGM of 96 h incubation. The transcription factor PDX-1 showed its highest transcription level at 48 h, and went slightly down until 168 h. INSULIN-1 and INSULIN-2 were positive on all analyzed samples, showing a slight expression decrease after 72 h of incubation and a recuperation expression after medium change. Those samples collected after medium change went down again and reach a lowest point at 168

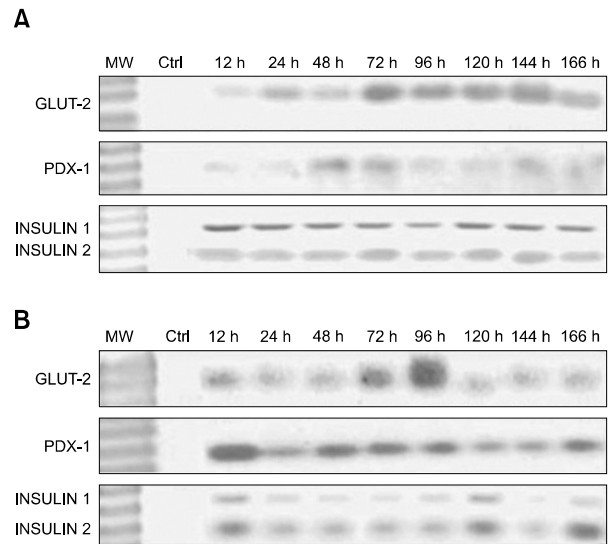
h. Samples obtained after 60 min of stimulation showed highest GLUT-2 expression at 96 h. PDX-1 were detected from 12 h of HGM change. INSULIN-1 exhibited less expression than INSULIN-2, nevertheless it was observed in all analyzed samples. Non of the control cultures showed a positive transcription for these genes (Fig. 2).

**Immunocytochemical Analysis of Lineage-Specific Markers**

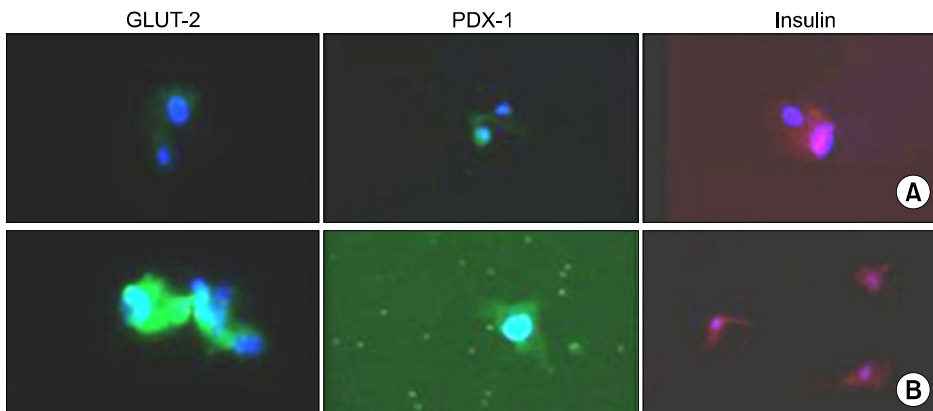
Immunocytochemistry imaging of cells incubated according to the induction protocol, showed positive labeling of GLUT-2, PDX-1 and INSULIN after 60 min of incubation with HGM (Fig. 3), in agreement with RT-PCR results (Fig. 3) Non of the control cultures showed a pos-



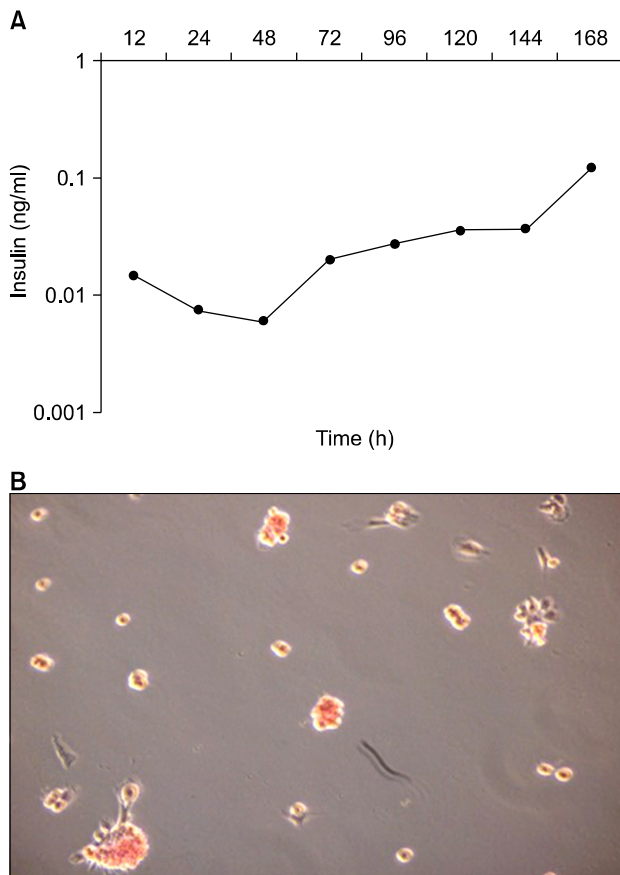
**Fig. 1.** Nestin expression after mesencymal stem cells incubation with neuro induction medium (NIM). (A) Representative images of agarose electrophoresis of RT-PCR products for NESTIN. Lane 1: molecular weight markers. Lane 2: cells grown in DMEM-F12 (Ctrl). Lanes 3-5: cells grown in neuro-induction médium after 2, 4 and 6 h of incubation with NIM. (B) Representative images of immunofluorescence detection of nestin by immunocytochemistry (Green) after 12 h of incubation with NIM. The nucleus is labeled with 4',6-diamidino-2-phenylindole (DAPI).



**Fig. 2.** Agarose electrophoresis of RT-PCR products for GLUT-2, PDX-1 and INSULIN 1,2 gene expression from mRNA samples of cell incubated with high glucose medium (HGM) for 12 h to 168 h. Samples were taken after 30 min (A) or 60 min (B) of glucose stimulation.



**Fig. 3.** Immunocytochemistry image for GLUT-2 (green), PDX-1 (green) and cytoplasmic insulin (red) of cells from culture incubated in high glucose medium (HGM), washed and stimulated. (A) after 30 min of incubation with glucose 25 mM and (B) after 60 min of glucose stimulation.



**Fig. 4.** Insulin detection. (A) Graphic representation of secreted insulin detected by EIA after mesenchymal stem cells pre-incubation in neuro differentiation medium (NIM) for 2 h and then incubated with high glucose medium (HGM). Measures were performed from samples obtained between 12 to 168 h. (B) Microphotography of cytosolic insulin detected by dithiozone staining after mesenchymal stem cells pre-incubation in (NIM) for 2 h and them incubated with HGM for 168 h.

itive mark for these proteins.

### Insulin detection

Samples collected from cells incubated in HGM at 12 to 168 h were used to perform EIA in order to detect secreted insulin. Results showed detectable values of secreted insulin consistent with 72 h of culture (Fig. 4A).

### Dithiozone staining

In order to verify the production of insulin by IPCs, dithiozone staining was used. Dithiozone binds to insulin and turns red. Staining red or brown indicates the presence of insulin granules in cell aggregates. Cells from culture incubated with HGM confirm Insulin-containing cells in spheroids after 168 h of incubation. The dithiozone positive cells were in the 80~90% range (Fig. 4B).

## Discussion

Nestin is a marker for pancreatic stem cells and for islet progenitor cells, playing an important role in the stemness and differentiation of stem cells into insulin-secreting cells (24-28). In pancreas, NESTIN expression is considered an intermediate regulator governing the proliferation and differentiation of new islet cells (20). The above explains why this protocol was first oriented towards NESTIN expression. Several protocols have described the induction of NESTIN expression on embryonic and adult stem cells, with the caveat that the process takes from 1 to 7 days (27, 29-32). The induction medium used on this research, was capable to promote MSCs to express NESTIN as early as 2 h. Nestin positive cells were important because they represent the first step to re-directed and activate signaling pathways that lead the expression of the PDX-1 gene. It is well known that PDX-1 is a transcription factor involved on embryonic development of the pancreas, and in the normal pancreatic islet of adult organisms, works regulating the expression of INSULIN and GLUT-2 among other genes.

PDX-1 is an important transcription factor expressed throughout the epithelium of early pancreatic buds and restricted to beta cells in the adult animal, where it plays a role in INSULIN expression and glucose response. Furthermore, exogenous PDX-1 expression during *in vitro* differentiation of embryonic cells clearly enhanced the expression of INSULIN. Based on those facts, the transfection of adult stem cells with vectors carrying the PDX-1 gene has been used to obtain IPCs (17, 19, 33-35), including nestin positive cells transfected with plasmids carrying rat Pdx-1 and BTC genes or PDX-1 mRNA allow to induce MSCs into the islet like cells (18-22).

Until now several protocols have been proposed to directly differentiate MSC into IPCs and although they have been successful, their approach takes more than 2 weeks and several steps to obtain IPCs (15, 31, 36-39). In 2012, there was a protocol developed by Her et al. (26), which included four steps to obtain Nestin positive cells from human blood derived stem cells incubated in a three-dimensional culture. These cells express nestin after eight days and then differentiate into IPCs. Our protocol proposes three short steps to obtain IPCs from rat bone marrow on less than 7 days. In fact, results show that cells incubated on differentiation medium as early as 24 h were capable to express INSULIN-1, INSULIN-2, PDX-1 and GLUT-2, after a glucose challenge. Based on these results and following the same scheme, we are proposing this protocol to be followed on stem cells from different sources,

to confirm the early response of nestin positive cells differentiation into IPCs precursors.

## Conclusion

The induction of mesenchymal stem cells to early express NESTIN and the following incubation on differentiation medium, allowed these cells to express INSULIN, PDX-1 and GLUT-2 genes in response to glucose stimulation. This precursor cells will be an important resource to cell replacement of progenitor pancreatic cells.

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## Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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