BMB
reports

Glucocorticoid treatment independently affects expansion and transdifferentiation of porcine neonatal pancreas cell clusters

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The purpose of this study was to determine the effects of duration and timing of glucocorticoid treatment on the expansion and differentiation of porcine neonatal pancreas cell clusters (NPCCs) into β-cells. After transplantation of NPCCs, the ductal cyst area and β-cell mass in the grafts both showed positive and negative correlations with duration of dexamethasone (Dx) treatment. Pdx-1 and HNF-3β gene expression was significantly downregulated following Dx treatment, whereas PGC-1α expression increased. Pancreatic duct cell apoptosis significantly increased following Dx treatment, whereas proliferation did not change. Altogether, transdifferentiation of porcine NPCCs into β-cells was influenced by the duration of Dx treatment, which might have been due to the suppression of key pancreatic transcription factors. PGC-1α plays an important role in the expansion and transdifferentiation of porcine NPCCs, and the initial 2 weeks following transplantation of porcine NPCCs is a critical period in determining the final β-cell mass in grafts. [BMB reports 2012; 45(1): 51-56]

INTRODUCTION

The prevalence of diabetes mellitus and chronic metabolic diseases is currently increasing worldwide. Until now, islet transplantation has been the only strategy to cure diabetes (1-3) in insulin-deficient diabetic patients. After the introduction of the Edmonton protocol, the clinical efficacy of islet transplantation was improved (4-6). However, the shortage of human sources of islets as well as the high frequency of immune rejection of transplanted tissue remain obstacles that need to be overcome.

Previously, we reported that porcine neonatal pancreatic cell clusters (NPCCs) contain many duct-like precursor cells and have considerable capacity for growth and transdifferentiation into β-cells compared to cells in the adult pancreas, making porcine NPCCs a useful alternative source of islets for transplantation (7-10). Clinical application of xenograft stem cells, such as transplantation of porcine NPCCs, still requires that the major obstacle of xenogenic immune rejection be overcome. Glucocorticoid is one of the most important and widely administered immune suppressants following organ transplantation. However, there are well-known deleterious effects of glucocorticoids on pancreatic β-cell function and β-cell expansion (11, 12). Furthermore, recent studies using a glucocorticoid receptor-inactivation model have suggested that excess glucocorticoid impairs differentiation of the β-cell lineage exclusively in the pancreas (13, 14). We have previously reported that the glucocorticoid analogue dexamethasone (Dx) suppresses the expansion and transdifferentiation of transplanted NPCCs into β-cells in normal nude mice (15). However, the molecular mechanisms underlying the effects of glucocorticoids on expansion and transdifferentiation of transplanted porcine NPCCs remain to be determined.

The peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) acts as a coactivator of several nuclear receptors as well as transcription factors (16-18). Recent studies have reported that PGC-1α plays an important role in the pathogenesis of β-cell dysfunction in type 2 diabetes (19), and moreover, PGC-1α modulates glucocorticoid receptor (GR) activity and is functionally involved in the GR-mediated transcriptional downregulation of insulin genes (20). Therefore, we hypothesized that Dx treatment might suppress the expansion and differentiation of porcine NPCCs into β-cells and that this is closely related to the expression of PGC-1α.

To test our hypothesis, we evaluated the effects of Dx treatment on the rates of expansion and transdifferentiation of porcine NPCCs after transplantation, and we analyzed the transcriptional regulation of β-cell-specific genes and GR-related genes following Dx treatment in monolayer-cultured porcine

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http://dx.doi.org/10.5483/BMBRep.2012.45.1.51

Received 9 May 2011, Revised 21 June 2011, Accepted 26 August 2011

Keywords: Dexamethasone (Dx), Diabetes mellitus, Neonatal porcine pancreas cell clusters (NPCCs), Pancreatic β-cells, Transdifferentiation

NPCCs. In this study, we determined that the cellular action mechanism of Dx in the regulation of expansion and differentiation of pancreatic precursor cells *in vivo* and vitro.

RESULTS

Total graft mass, β-cell mass, and relative volume of β-cells in graft according to time and duration of Dx treatment

To elucidate the effects of time and duration of Dx treatment, an early treatment (ET) group was treated daily with Dx only during the first 2 weeks, and a late treatment (LT) group was treated daily with Dx during the last 8 weeks of the 10-week treatment period after transplantation. Total graft mass decreased significantly in the Dx-treated group, regardless of treatment duration (Fig. 1A). However, the relative volume of β-cells in the graft significantly decreased in the LT and continuous treatment (CT) groups, but not in the ET group (Fig. 1B). The total β-cell mass was significantly lower in all Dx-treated groups compared to the control group. In contrast, the relative volume of the grafts' ductal cysts increased significantly with the duration of Dx treatment, reaching 29% of the graft volume in the CT group (Fig. 1C). In control mice, the graft tissue consisted of more than 40% insulin-stained β-cells and duct cell clusters with a small number of ductal cysts. In contrast, numerous ductal cysts were observed in the grafts of the LT and CT groups (Fig. 1D). These cystic structures were abundant in all Dx-treated groups. The area of the ductal cyst increased with Dx treatment in a duration-dependent manner.

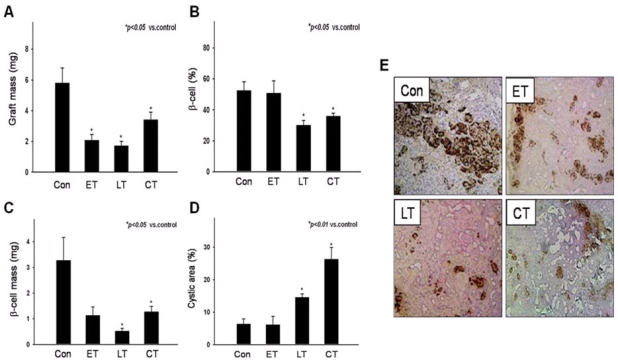

Fig. 1. Total graft mass, β-cell mass, and relative volume of β-cells in the graft according to time and duration of Dx treatment. Porcine NPCCs transplanted under the kidney capsule of normal nude mice. Total graft mass (A), b-cell percentage (B), and absolute β-cell graft mass (C) decreased significantly in the Dx-treated group in comparison with the non-treated group. The cystic area in the CT group significantly increased as compared to the control group and another Dx-treated group (D). Morphologic analysis of porcine NPCCs was performed on the graft at 10 weeks after transplantation (E). Control graft tissue comprised β-cells (brown color) and some duct cells, but 10 weeks after Dx treatment, the graft tissues comprised profuse duct-like cystic structures and a few β-cells (200×) (*P < 0.05, Scale bar = 40 μm). Con, control; ET, early treatment; LT, late treatment; CT, continuous treatment.

Conversely, the number of insulin-stained β-cells markedly decreased, especially in the CT group (Fig. 1E).

Expression of transcription factors in the graft after Dx treatment

The expression of Pdx-1 and HNF-3β mRNA in the grafts and monolayer-cultured porcine NPCCs was examined after Dx treatment for 48 h. The expression of transcription factors related to pancreatic development, including Pdx-1 and HNF-3β, decreased significantly in the grafts after Dx treatment. The expression of PGC-1α, which is a well-known coactivator that binds to glucocorticoid receptor, did not significantly change in the grafts (Fig. 2A and 2C). Expression of the Pdx-1 and HNF-3β genes was downregulated by Dx treatment for 96 h. However, PGC-1α gene expression increased, which was inversely related to Pdx-1 expression (Fig. 2B and 2D).

Characterization and quantification of cell components of monolayer-cultured porcine NPCCs in culture dishes after Dx treatment

To characterize the cell population of cultured porcine NPCCs after Dx treatment, we performed immunostaining. The number of pan-CK-stained duct cells decreased upon Dx treatment, whereas the number of vimentin-positive mesenchymal cells increased (Fig. 3A). The number of endocrine hormone-positive cells did not significantly change with short-term Dx treatment (Fig. 3B). Immunofluorescence staining of PGC-1α, insulin, and Pdx-1 was performed in monolayer-cultured porcine NPCCs after Dx treatment for 96 h (Fig. 3C). The number of PGC-1α, positive cells increased in the

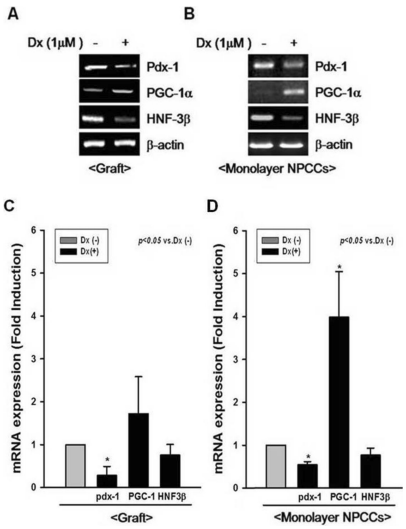

Fig. 2. Expression of transcription factors in the graft after Dx treatment. The expression of pancreatic transcription factors was determined at 1 day after transplantation in the graft (A, C) and 96 h after plating monolayer-cultured porcine NPCCs (B, D) (*P < 0.05).

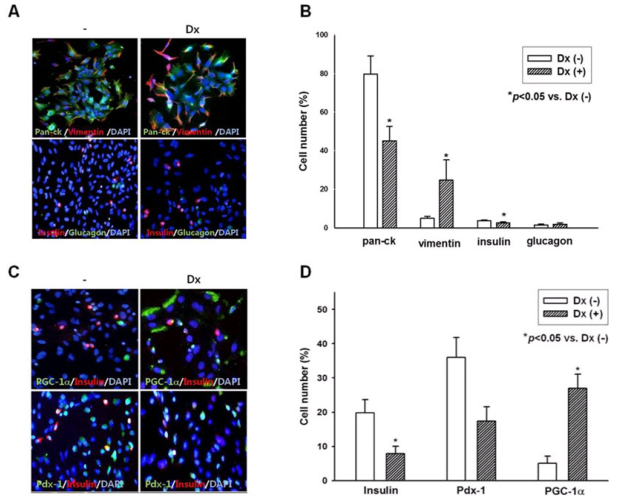

Fig. 3. Characterization and quantification of cell components of monolayer-cultured porcine NPCCs after Dx treatment. Immunofluorescence of pan-cytokeratin (pan-CK), vimentin, insulin, and glucagon were performed on monolayer-cultured porcine NPCCs (A). In the Dx treatment group, the percentage of pan-CK- and vimentin-positive cells increased significantly as compared to the non-Dx-treated group (B). Double immunofluorescence staining of PGC-1α with insulin or Pdx-1 with insulin was performed on monolayer-cultured porcine NPCCs (C). The number of PGC-1α-positive cells increased remarkably in the Dx-treated group, whereas the number of insulin-positive cells decreased after 48 h in culture (D). Pdx-1-positive cells also evidently decreased with Dx treatment (*P < 0.05, Scale bar = 40 μm).

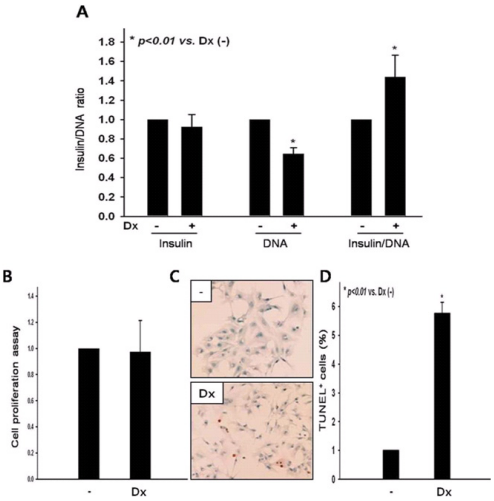

Fig. 4. Insulin content, proliferation, and apoptosis in monolayer-cultured porcine NPCCs. The insulin and DNA contents were measured at 96 h after plating of monolayer-cultured porcine NPCCs (A). The insulin content did not differ significantly, but the DNA content significantly decreased in the Dx-treated groups as compared to the non-Dx treated group. Cell proliferation did not change after Dx administration in either group. TUNEL staining of monolayer-cultured porcine NPCCs (B). In monolayer-cultured porcine NPCCs, Dx treatment (1 μM) significantly induced apoptosis (brown) as compared with the non- Dx-treated group (C), and the percentage of TUNEL-positive cells in the Dx-treated group significantly increased in comparison with the non-Dx-treated group (D) (*P < 0.05, Scale bar = 40 μm).

Dx-treated group as compared to the non-treated group, whereas the number of insulin- and Pdx-1-stained cells significantly decreased with Dx treatment (Fig. 3D)

Insulin content, proliferation assay, and apoptosis in monolayer-cultured porcine NPCCs

Insulin content was measured in homogenized monolayer-cultured porcine NPCCs (Fig. 4A). The insulin content did not differ significantly, regardless of Dx treatment. However, the DNA content significantly decreased in the Dx-treated group, and therefore, the insulin content/DNA ratio markedly increased. The duct cell proliferation rate did not differ, regardless of Dx treatment, although the number of duct cells decreased significantly after Dx treatment (Fig. 4B). However, the rate of apoptosis of the monolayer-cultured porcine pancreatic duct cells increased more than 5-fold after Dx treatment (Fig. 4C and 4D).

DISCUSSION

We have previously shown that Dx impairs the expansion and transdifferentiation of transplanted porcine NPCCs into insulin-secreting β-cells (15). In the present study, we performed *in vivo* experiments to determine whether or not the adverse effects of Dx on the expansion and maturation of porcine NPCCS are related to the duration or a specific time point of Dx treatment. For this, we analyzed the rate of proliferation and apoptosis of pancreatic duct cells as acting pancreas adult stem cells and evaluated the expression of pancreas-specific transcription factors in monolayer-cultured porcine neonatal pancreatic cells.

Porcine NPCCs are comprised of approximately 70% duct cells, 20% endocrine cells, and some fibroblasts. Since porcine NPCCs markedly expand and transdifferentiate into β-cells after transplantation, they are an effective ex vivo model for studying the development and regeneration of the pancreas (21). Here, we demonstrated that only 2 weeks of early Dx treatment caused a marked decrease in total graft mass after transplantation, suggesting that the expansion of the graft was determined at an early critical period after transplantation. We also observed that stem cell differentiation into mature duct cells, formation of ductal cysts, and transdifferentiation into β-cells were dependent upon duration of Dx treatment. Cell proliferation assay and immunostaining showed that Dx treatment disturbed the expansion of the full graft mass and delayed β-cell differentiation. Most of the grafts in the Dx-treated group comprised profound cystic components, although these did not arise from ductal cell proliferation but rather resulted

from increased ductal cell apoptosis after Dx treatment. However, transdifferentiation into β-cells was suppressed, and the cystic area inversely increased with increasing duration of Dx treatment. This suggests that the effects of glucocorticoids on the differentiation fate of pancreatic progenitor cells depend on the duration of Dx exposure.

The direct effect of glucocorticoids on pancreatic β-cell development has been described in an animal model of selective inactivation of the glucocorticoid receptor gene in pancreatic precursor cells (22, 23). We obtained similar results, which confirm the key role of glucocorticoids in the differentiation of pancreatic precursor cells. Our acute *in vivo* treatment experiment found that glucocorticoid suppressed the mRNA expression of pancreas-specific transcription factors, including Pdx-1 and HNF-3β. These results were reproduced in Dx-treated, monolayer-cultured porcine NPCCs. Interestingly, the decreased expression of Pdx-1 and HNF-3β closely correlated with the increased expression of PGC-1α in monolayer- cultured porcine NPCCs after Dx treatment. Decreased expression of insulin and Pdx-1 as determined by immunofluorescence staining of monolayer-cultured porcine NPCCs also correlated with the increased expression of PGC-1α. These findings were sufficient to confirm that PGC-1α is associated with the transcriptional regulation of pancreatic cell differentiation from pancreatic precursor cells.

In summary, Dx treatment remarkably suppressed graft mass expansion at an early stage following transplantation, and endocrine differentiation of porcine NPCCs correlated with the duration of Dx treatment. This impairment of β-cell differentiation appeared to be mediated by pancreatic transcription factors, which might be related to the expression of PGC-1α upon Dx treatment. Finally, we were able to demonstrate that the expression of PGC-1α was a crucial factor in glucocorticoid-suppressed expansion and transdifferentiation of porcine NPCCs.

MATERIALS AND METHODS

Materials

Antibodies specific to insulin, pan-cytokeratin (pan-CK), vimentin, and glucagon were purchased from Zymed (San Francisco, CA, USA). Anti-PGC-1α (sc-5816) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and anti-Pdx-1 was a gift from Dr. Kaneto Hideaki (Osaka University Graduate School of Medicine, Osaka, Japan). Trizol Reagent and SuperScript™II RT were purchased from Invitrogen (Carlsbad, CA, USA). Cell culture medium was purchased from GIBCO (Grand Island, NY, USA), and collagenase P was obtained from Boehringer-Mannheim (Indianapolis, IN, USA). An insulin radioimmunoassay (RIA) kit was purchased from Linco (Charles, MO, USA). All other chemicals were purchased from Sigma-Aldrich (Louis, MO, USA).

Experimental animals

Neonatal pigs aged 1-3 days were purchased from Sunjin (Icheon, Kyonggido, Korea). Male Balb/c nude mice were purchased from Charles River Laboratories (Orientbio, Seoul, Korea). The Animal Care Committee of the Catholic University of Korea approved the experimental protocols for this study, and all procedures conducted in this study were in accordance with the established ethical guidelines for animal studies.

Preparation of porcine NPCCs and transplantation

Porcine NPCCs were isolated from 1-3-day-old neonatal pigs as previously described (24). After 7 days of free-floating in culture, about 4,000 islet equivalents from the porcine NPCCs were transplanted under the kidney capsule of normoglycemic nude mice using a micromanipulator syringe, as described by Yoon *et al*. (21). To culture NPCC cells in a monolayer, the cell clusters were broken up by gentle aspiration, and 1.5×10^6 cells were cultured in DMEM supplemented with 10% fetal bovine serum in a humidified chamber containing 5% CO_2.

Dx treatment

From the day of transplantation, Dx (1 mg/kg) was injected intraperitoneally daily for 10 weeks in the continuous treatment (CT) group (n = 8); vehicle was injected at the same times in the control group (n = 8). To elucidate the effects of time and duration of Dx treatment, an early treatment (ET) group (n = 5) was treated with Dx only during the first 2 weeks, and a late treatment (LT) group (n = 5) was treated with Dx only during the last 8 weeks of the 10-week treatment period after transplantation. To analyze the effects of Dx on pancreas-specific transcription factors in the grafts, we injected Dx at 12 h intervals in nude mice, beginning 12 h before transplantation. For *in vitro* experiments, monolayer-cultured porcine NPCCs were treated with Dx (1 μM) beginning 3 days after dispersion and were harvested 96 h after Dx treatment.

Measurement of cellular insulin content

Cellular insulin content was analyzed in homogenized, harvested cultured porcine NPCCs, from which insulin was extracted with 1 ml of acidified ethanol solution. Insulin was quantified using a radioimmunoassay kit. DNA content was analyzed using a QIAamp® DNA Mini kit (QIAGEN, Valencia, CA, USA). The total intracellular insulin was normalized to the total DNA content.

Immunohistochemical staining of porcine NPCCs

Before staining, porcine NPCCs were fixed with 4% paraformaldehyde for 15 min at 4°C and then washed with 1× phosphate-buffered saline (PBS). To block non-specific binding sites, normal donkey serum was applied at a dilution of 1 : 100 for 30 min. Porcine NPCCs were incubated overnight at 4°C with primary antibodies: polyclonal rabbit anti-human pan-CK (1 : 100) with either polyclonal rabbit anti-human vimentin (1 : 200), monoclonal guinea pig anti-human insulin

(1 : 100), or polyclonal rabbit anti-human glucagon (1 : 100). The cells were then incubated with rhodamine-conjugated anti-mouse and FITC-conjugated anti-rabbit secondary antibodies for 1 h at room temperature, washed, and mounted with an anti-fade medium. Antibody labeling of PGC-1α (1 : 100) and Pdx-1 (1 : 2,000) was detected using the avidin-biotin complex (ABC) method with the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA) and streptavidin FITC-conjugated secondary antibodies.

Morphologic analysis of transplants

All porcine NPCC recipients underwent nephrectomy of the graft-bearing kidney for morphologic analysis. Graft weight was measured, and the tissues were prepared for immunostaining using guinea pig anti-human insulin antibody to quantify β-cell mass by the point-counting method (22). Insulin staining was amplified using the biotin-streptavidin method (Vector Laboratories) and developed with diaminobenzidine tetrahydrochloride. The volume of the cystic space in the graft was measured by planimetry using an image analyzer (Optimas 6.51, Media Cybertics, Tempe, AZ). The volume of the ductal cyst is presented as a percentage of the total graft tissue, calculated by dividing the area of the cystic structure by the total graft area and multiplying by 100. The β-cell mass of the graft was calculated by multiplying the relative percentage of β-cells by the total graft weight.

TUNEL analysis

Cell apoptosis was identified with the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection kit (Chemicon International Inc., Temecula, CA, USA). After dewaxing, cells were treated with proteinase K. This was followed by incubation with terminal deoxynucleotidyltransferase (TdT) enzyme solution. The reaction was terminated by incubation in stop/wash buffer. The cells were incubated with anti-digoxigenin (DIG) peroxidase, and then incubated with diaminobenzidine and 0.01% H_2O_2.

Cell proliferation assay

Cell proliferation was quantified using Counting kit-8 (CCK-8, Dojindo Laboratories, Tokyo, Japan) in 96-well microplates. Briefly, 10 μl of CCK-8 solution was added directly to the cell culture medium after the cells had been treated with Dx for the indicated time. The plate was then incubated for 1-4 h, and the absorbance at 450 nm was measured with a microplate reader.

RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

For RT-PCR, total RNA was obtained from grafts and monolayer-cultured porcine NPCCs using Trizol, according to the manufacturer's instructions. Two micrograms of RNA was reverse-transcribed into cDNA using oligo$(dT)_{12\text{-}18}$ primers with SuperScript™II at 42°C for 1 h, followed by 72°C for 15 min in standard buffer. The PCR primer sequences were as follows (forward, reverse): PGC-1α: 5'-CCTTTCTGAACTTGATGTGA-3', 5'-ATGCTCTTTGCTTTATTGCT-3'; Pdx-1: 5'-AAGGCTCACGC GTGGAAAAGG-3', 5'-CATGCGGCGGTTTTGGAACC-3'; HNF-3β: 5'-GGAGCGGTGAAGATGGAAG-3', 5'-TACGTGTTCATG CCGTT CAT-3'; β-actin: 5'-ATCATGTTTGAGACCTTCAACAC CC-3', 5'-CATGGTGGTGCCGCCAGACAG-3'. The amplification was repeated for 30 cycles of 94°C for 30 s, 52-60°C for 1 min, and 72°C for 30 s. The PCR products were visualized by 1.5% agarose gel electrophoresis. The density of each band was measured using a VDS densitometer (Pharmacia Pharmacia Biotech, Uppsala, Sweden).

Statistical analysis

All groups were statistically compared with a Kruskal-Wallis rank test to detect any differences with respect to time and duration of Dx treatment. Post-hoc Tukey comparison tests were performed to identify significant differences between the groups. Statistical analysis was performed using Statistical Analysis System (SAS) software (release 8.12, SAS Institute, Cary, NC, USA). A null-hypothesis probability of <0.05 was considered significant.

Acknowledgements

This work was supported by a grant from the Ministry of Health & Welfare, Republic of Korea (A040004) and by a grant from the Korea Healthcare technology R&D Project (A092258) funded by the Ministry of Health, Welfare & Family Affairs, Republic of Korea.

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