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2,4 -Dinitrophenol Trans-Differentiate NIH3T3 Cells into Insulin Producing β-Cells

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ABSTRACT

Diabetes is one of the most common diseases worldwide. Type I diabetes is characterized by the degeneration of β-cells. The present study is an attempt in the search of a suitable and efficient way to enhance the transdifferentiation ability of mature fibroblasts by using transgene free cellular approach in order to improve the cell-based therapeutics for diabetes. In the present study, we analyzed the effect of 2, 4 - Dinitrophenol (DNP) on the transdifferentiation of NIH3T3 cells into insulin producing β-cells (IPCs). DNP is a lipophilic weak acid that uncouple oxidative phosphorylation by decreasing ATP production. Cells treated with DNP were analyzed for the morphological changes and islet specific markers (MafA, Ngn3, Nkx 6.1, Pdx-1, insulin, glucagon, somatostatin and Sca-1) at gene and protein levels. In the presence of DNP, NIH3T3 cells appeared round, small and contracted while after reoxygenation they regained the normal fibroblast like spindle shaped morphology. The strategy to induce efficient transdifferentiation of NIH3T3 cells into IPCs has shown positive endocrine expression pattern, specifically β-cell specific transcription factors, demonstrating their successful regeneration. It is concluded that DNP has a potential to induce efficient transdifferentiation of NIH3T3 cells into insulin producing β-cells. The study could further be evaluated for their *in vivo* effect and serve as an improved and effective cellular therapeutic option for type 1 diabetes. It may offer the possibility of improved regeneration of damaged β-cells from mature cell type to functional insulin producing β-cells that could serve as a future therapeutic approach for diabetes.

INTRODUCTION

Type 1 diabetes (T1D) that causes substantial morbidity and mortality, generally fallouts from the autoimmune destruction of pancreatic β-cells, resulting in the elevated blood glucose levels (Atkinson *et al*, 2014). In order to produce insulin to maintain normoglycemia, the lost β-cells must be replenished (Melton, 2011). Exogenous insulin administration is the only effective management of blood glucose levels. However, insulin treatment comes along with the hypoglycemic episodes and does not eliminate the chronic complications associated with diabetes, like cardiovascular complications, nephropathy, neuropathy, retinopathy and often death (Gerace *et al*., 2014). Several anti-diabetic drugs including metformin, sulfonylureas, glucagon-like peptide 1 agonists, thiazolidinediones, α-glucosidase inhibitors, and dipeptidyl peptidase-4 inhibitors are available to regulate blood glucose level. However, their continuous administration leads to several pathological outcomes including hypoglycemic episodes, ketoacidosis as well as micro and major complications affecting nervous, retinal, renal, cerebrovascular, and cardiovascular systems (Pandian *et al*., 2014).

Other therapeutic approach includes pancreas and islet transplantation, though this treatment is hampered because of limited cadaveric donors and the use of lifetime immunosuppressants (Johannesson *et al*., 2015). The cell replacement therapy constitutes one of the best approaches to target T1D, as it is caused by the destruction of β-cells (Borowiak and Melton, 2009). In the last few years, several promising approaches have been suggested for β-cell regeneration, including reprogramming of non β-cells, direct differentiation of stem cells or trans-differentiation of mature somatic cells, like pancreatic duct cells and hepatocytes into pancreatic β-like cells, thus giving new insight to cure T1D (Lemper *et al*., 2014).

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A transgene-free cellular reprogramming has shown efficient regeneration of a desired cell type by using small molecules. Moreover, this transgene free reprogramming with small molecules is non immunogenic, easier and more conventional than the virus mediated reprogramming approach (Masuda *et al*., 2013). Small molecules, like the epigenetic enzyme inhibitors and signalling pathway modulators, target key transcription factors, and enhance the direct or indirect differentiation of stem cells or mature cell types into functional insulin producing cells (IPCs) (Pandian *et al*., 2014). Several small molecules that activate or inhibit the epigenetic enzymes could enhance the differentiation potential, for example, 5-aza-2′-deoxycytidine (5-AZA), a DNA methyl transferase inhibitor, triggers Ngn3 expression and promotes endocrine cell differentiation in the human pancreatic ductal cell line (PANC-1) (Lefebvre *et al*., 2010). XW4.4, an aminopyrrole derivative has been shown to differentiate MSCs into IPCs via HNF3b (hepatocyte nuclear factor 3b) induction (Ouyang *et al*., 2014). Moreover, combined treatment of selenite, 5-AZA, retinoic acid and trichostatin A (TSA), chromatin remodelling regulator proteins like insulin and transferrin resulted in direct differentiation of insulin producing cells from rat liver epithelial stem-like WB-F344 cells (WB cells) (Liu *et al*., 2013).

In the present study, we analyzed the effect of DNP on the transdifferentiation of NIH3T3 cells into insulin producing β-cells. DNP is a lipophilic weak acid that uncouple oxidative phosphorylation by decreasing ATP production (Shavell *et al*., 2012). Cells treated with DNP were analyzed for the morphological changes and islet specific markers (MafA, Ngn3, Nkx 6.1, Pdx-1, insulin, glucagon, somatostatin and Sca-1) at gene and protein levels.

The choice of defined transcription factors and islet specific markers hold key to efficient transdifferentiation. Some of these factors play important roles in pancreatic beta cell development. MafA is a basic leucine zipper, the homologue of v-Maf oncoprotein and belongs to musculoaponeurotic fibrosarcoma oncogene family. It is expressed in the initial stages of β-cell production and involved in insulin gene expression. It is the principal transcription factor for β-cell development, maturation, reprogramming, production and maintenance of insulin producing cells. MafA is considered a potent transactivator of insulin gene (Matsuoka *et al*., 2007). During pancreas development, MafA expression is first detected at the beginning of the principal phase of insulin-producing cell production. Neurogenin3 (Ngn3) belongs to basic helix loop helix transcription factor family, and is known to play an important role in pancreatic development and endocrine differentiation (Dominguez *et al*., 2005). Ngn3 is also involved in the regulation of a variety of pancreatic transcription factors such as NeuroD, Pax4 and Nkx2.2 (Watada *et al*., 2003). Early pancreatic marker, Sca-1 is co-expressed with Pdx-1 and Ngn3 (Ma *et al*., 2012). Sca-1 is expressed especially in islets and ductal cells (Seaberg *et al*., 2004).

The present study is an attempt in the search of a suitable and efficient way to enhance the transdifferentiation ability of mature fibroblasts by using transgene free cellular approach in order to improve the cell based therapeutics for diabetes.

MATERIALS AND METHODS

Cell culture

Mouse embryonic fibroblast cell line, NIH3T3 was purchased from American Type Culture Collection (ATCC) by the Bio-bank facility of the Dr. Panjwani Center for Molecular Medicine and Drug Research (PCMD). The cells were seeded in high glucose Dulbecco's modified Eagle's medium/F12 (DMEM/F12) supplemented with 20% fetal bovine serum (FBS); 100 units/mL penicillin and 100 µg/mL streptomycin.

DNP treatment

70% confluent NIH3T3 cells were treated with 2, 4 dinitrophenol (DNP). To find out optimal concentration of DNP, cells were treated with different concentrations of DNP (0.025-2 mM) for 10 and 20 min. The optimal concentration of 0.1 mM for 20 min was selected on the basis that cells at this concentration only experienced temporary shock. Initially, medium was aspirated and cells were washed twice with incomplete medium. 0.1 mM DNP with FBS free medium was added for 20 min and cells were incubated at 37 °C. After 20 min, medium was aspirated and cells were washed with incomplete medium. Finally, cells were reperfused in the presence of complete medium for 48 h in CO_2 incubator.

Morphological examination of DNP treated cells

After DNP treatment and 48 h of reperfusion, cells were analyzed for morphological changes and compared with that of untreated control.

Analysis of pancreatic genes in DNP treated cells

Total RNA was isolated using SV total RNA system kit (Promega, USA) by spin method according to manufacturer's instructions. 1 µg of the isolated RNA was subjected to cDNA synthesis by using SuperScript III first-strand synthesis kit (Invitrogen Life Technology, USA). Primer 3 design program was used to design primers for each gene at http://frodo.wi.mit.edu/primer3/, and purchased from Integrated DNA technologies (IDT,

USA). Gene expression analysis of islet specific genes, insulin, somatostatin, Neurogenin3 (Ngn3), glucagon, NK6 homeobox 1 (Nkx 6.1) and MafA was performed by RT-PCR (Table I). DNA was amplified with initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation, annealing, and extension at 95°C, 58-64°C and 72°C, respectively, and then final extension at 72°C for 10 min. The amplified products were stored at -20°C until further use. GAPDH was used as positive control.

Analysis of pancreatic proteins in DNP treated cells

After 48 h reoxygenation, cells were dissociated with cell dissociation solution, centrifuged and analyzed for the expression of pancreatic proteins by flow cytometry using the protocol described by Haneef *et al*. (2014). Primary antibodies against insulin, MafA, Ngn3, Pdx1, glucagon and Sca1 were used at 1:40 dilution. Alexa fluor goat anti-mouse or anti-rabbit secondary antibody at 1:500 dilution was used for detection. Unlabelled cells or cells labelled with the secondary antibody were used as control. Labelled cells were observed in FL-1 filter. FSC was selected as threshold parameter and threshold was set to a value of 52 which eliminate small debris.

Statistical analysis

Significance of difference among the groups was analyzed by using One-way ANOVA. The results were illustrated as mean ± S.E.M. P-value <0.05 was considered statistically significant. Analysis was done by using SPSS program (version 13, SPSS Inc, Chicago, IL, USA).

RESULTS

Morphological characteristics

NIH3T3 cells treated with different concentrations of DNP (0.025 mM – 2 mM) for 20 min showed round and contracted morphology as compared to the untreated control. After 48 h of reoxygenation, cells regained normal morphology at all concentrations except in case of 1 mM and 2 mM at which most of the cells died (Fig. 1A-B). We selected 0.1 mM concentration of DNP for 20 min for all subsequent experiments as cells only shrunk at this concentration and their normal morphology was restored after reoxygenation.

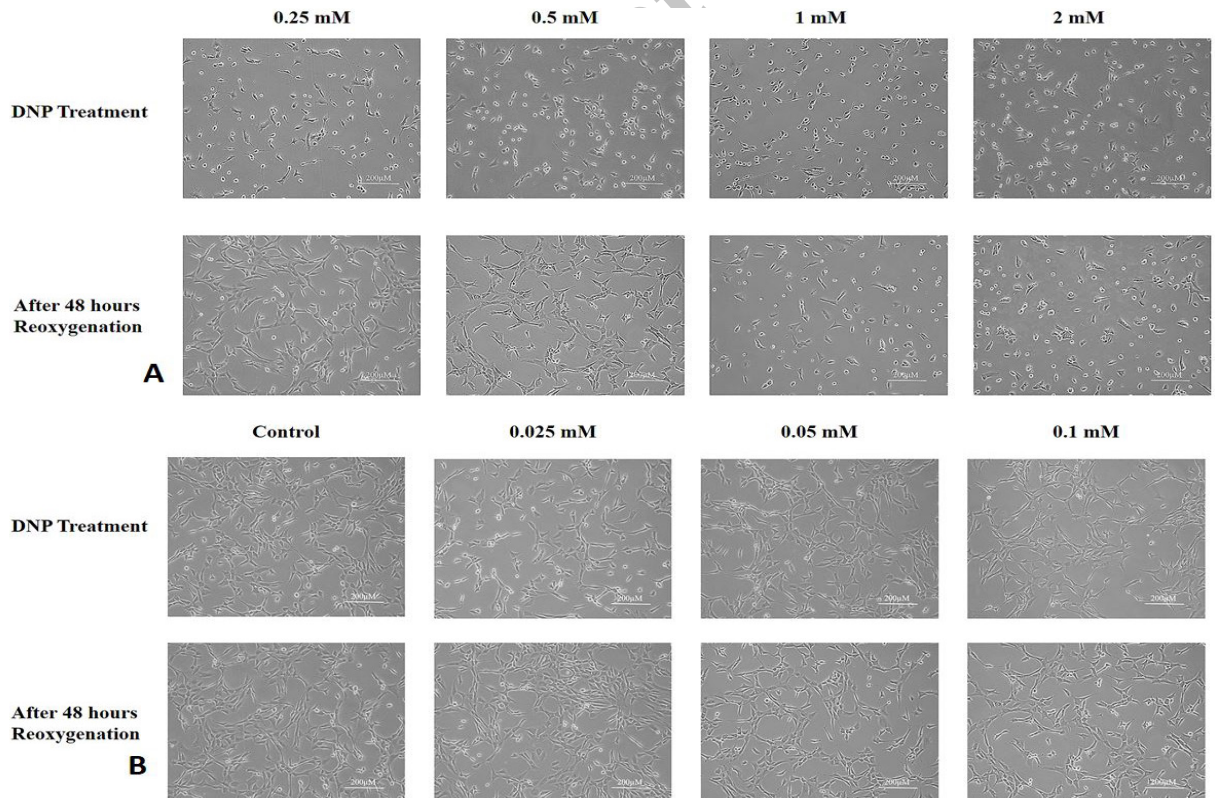

Fig. 1. Morphology of NIH3T3 cells after DNP treatment. NIH3T3 cells treated with different concentrations of DNP (0.025 mM – 2 mM) for 20 min were round and shrunken. After 48 h of reoxygenation, cells regained normal morphology except in case of 1 mM and 2 mM concentrations in which most of the cells died (Fig. 1A-B). Images were taken at 10X magnification under inverted phase contrast microscope.

Table I. Forward and reverse primer sequences, accession numbers, annealing temperatures and expected product sizes.

Gene PCR primer	Accession No.	Primer Sequence (5'- 3')	Annealing temperature (°C)	Product size (bp)
GAPDH	BC09593	F GGAAAGCTGTGGCGTGATGG R GTAGGCCATGAGGTCCACCA	60	414
Insulin	NM_008386	F CCACCCAGGCTTTTGTCAAG R TCCAGCTGGTAGAGGGAGCA	64	251
MafA	NM_194350	F ATCATCACTCTGCCCACCAT R AGTCGGATGACCTCCTCCTT	58	208
Somatostatin	NM_0092151	F CCCCAGACTCCCGTCAGTTTC R TGGGTTCGAGTTGGCAGACC	63	200
Ngn3	NM_009719	F GAGTTGGCACTCAGCAAAC R TCTGAGTCAGTGCCCAGAT	56	350
Glucagon	NM_008100	F CCCAGACAGAAGCGCATGAG R GAATTCCTTTGCTGCCTGGCC	63	251
Nkx 6.1	NM_144955.2	F TCAGGTCAAGGTCTGGTTCC R CGATTTGTGCGTTTTTCAGCA	56	211

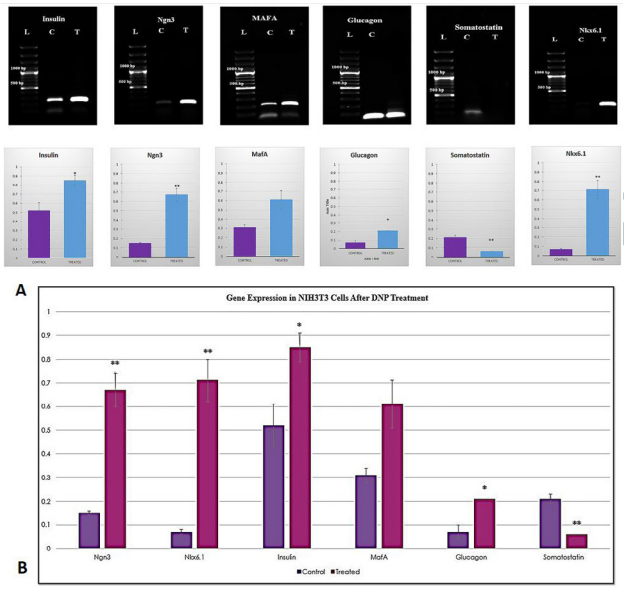

Fig. 2. RT-PCR analysis of pancreatic genes in NIH3T3 cells after DNP treatment. Expression levels of insulin, Ngn3, MafA, Nkx6.1 and somatostatin in NIH3T3 cells before and after DNP treatment. Graphical representation is also shown. Ngn3, Nkx 6.1, insulin, and glucagon expression was significantly increased ($p < 0.01$, $p < 0.01$, $p < 0.05$ and $p < 0.05$, respectively) while that of somatostatin was significantly decreased ($p<0.001$) after DNP treatment. No significant change was observed in MafA expression after DNP treatment (Fig. 2A). Combined graphical representation of pancreatic gene expression in untreated and DNP treated groups is also shown (Fig. 2B). Data is presented as mean ± S.E.M; level of significance is $p < 0.05$; (where *** = $p < 0.001$, ** = $p < 0.01$, and * = $p < 0.05$.

Pancreatic genes expression after DNP treatment

To check the effect of DNP on transdifferentiation of NIH3T3 cells, pancreatic transcription factors (MafA, Ngn3, Nkx 6.1) and pancreatic genes (insulin, glucagon, somatostatin) were analyzed at mRNA level by RT-PCR. Treated cells expressed insulin, MafA, and Ngn3 whereas somatostatin expression was found to be down regulated after DNP treatment (Fig. 2A). Significant increase in the expression of insulin, glucagon ($p< 0.05$), Ngn3 and Nkx6.1 ($p<0.01$) while significantly decreased ($p< 0.05$) expression of somatostatin was observed after DNP treatment. No significant change was observed in MafA expression levels as compared to untreated control (Fig. 2B).

Pancreatic proteins expression after DNP treatment

To evaluate the effect of DNP on the differentiation of NIH3T3 cells, expression of pancreatic proteins, insulin, glucagon, MafA, Ngn3, Pdx-1, and Sca1 were analyzed by flow cytometry (Fig. 3A). Treated NIH3T3 cells showed significant increase ($p <0.001$) in insulin, MafA, Pdx1, glucagon and Ngn3 expressions as compared to untreated control (Fig. 3B).

DISCUSSION

Oxygen plays a critical role in maintaining cellular homeostasis, regulation of stem cells function and development of several organs, including pancreas (Shah *et al.*, 2011). Typically, in normal cells oxygen deficiency contributes in the cell death, while in stem cells it controls cellular properties including self-renewal and differential potential by regulating signalling pathways and expression

of key regulatory transcription factors (Hakim *et al*., 2014). It has been known to control differentiation of various cell types including pancreatic cells (Fraker *et al*., 2007) neural cells (Freitas-Correa *et al*., 2013) and cardiomyocytes (Khan *et al*., 2016).

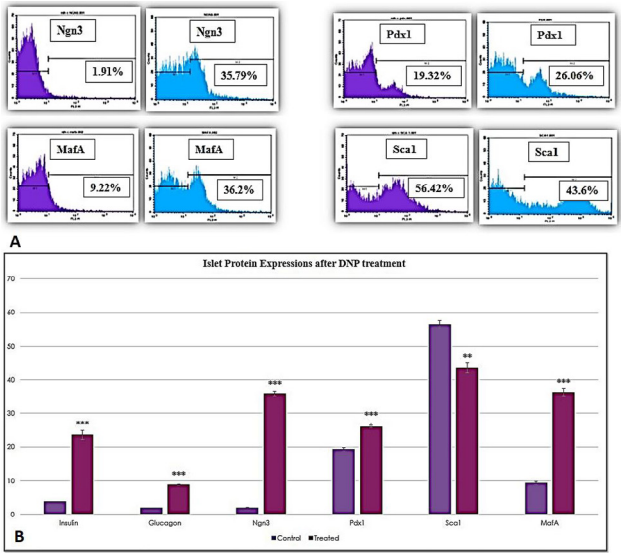

Fig. 3. Flow cytometric analysis of pancreatic proteins in NIH3T3 cells after DNP treatment: pancreatic proteins, MafA, Pdx1, insulin, glucagon, Ngn3 and Sca1 were analyzed in NIH3T3 cells before and after treatment with DNP. Cells labelled with Alexa fluor 546 goat anti-mouse or anti-rabbit secondary antibodies were used as controls (Fig. 3A). Number of positive cells are shown as percentage of untreated cells. Significant increase ($p<0.001$) in insulin, glucagon, MafA, Ngn3 and Pdx1 expressions was observed after DNP treatment (Fig. 3B). Data is presented as mean ± S.E.M.; level of significance is $p < 0.05$; where *** = $p < 0.001$, ** = $p < 0.01$, and * = $p < 0.05$.

In the present study DNP was used to analyze its role in the trans differentiation of mouse embryonic fibroblasts to insulin producing beta cells. DNP is a metabolic inhibitor, it uncouples the oxidative phosphorylation by decreasing ATP production (Shavell *et al*., 2012). It is commonly used to chemically induce the low oxygen environment and metabolic stress in different cell types (Khan *et al*., 2016). Earlier our group has reported the role of DNP in cell survival and angiogenic genes induction (Ali *et al*., 2015), increased fusion potential of cardiomyocytes and rat MSCs (Haneef *et al*., 2014). We further reported that preconditioning of bone marrow derived mesenchymal stem cells with DNP increased expression of angiogenic factors including Angiopoietin 1, erythropoietin, VEGF and FGF (Ali *et al*., 2015). VEGF is a hypoxia marker and is found to be involved in the process of angiogenesis (Han *et al*., 2016).

In this study the effect of DNP on the expression of pancreatic genes and proteins in the NIH3T3 cells was analysed. We optimized DNP concentration by treating cells with different concentrations (0.025 – 2 mM) followed by 48 h of reperfusion. DNP treated cells appeared small and shrunken, displaying stress condition. However, they attained normal morphology after reoxygenation. The concentration of 0.1 mM was selected on the basis of having lesser number of apoptotic cells. After treatment, expression of almost all pancreatic genes was upregulated in NIH3T3 cells. Ngn3, Nkx6.1, insulin, and glucagon expressions were significantly increased ($p<0.01$, $p<0.01$, $p<0.05$ and $p<0.05$, respectively). MafA expression was also increased but the change was not significant. In contrast, somatostatin was significantly decreased ($p<0.001$) after DNP treatment. Also, protein concentrations of insulin, MafA, glucagon, Pdx1 and Ngn3 were significantly increased ($p<0.001$) after DNP treatment.

The enhanced expression of endocrine markers, specifically β-cell specific transcription factors demonstrate the successful regeneration of insulin producing β-cells. Furthermore, significant change in glucagon expression suggests that the cells are also differentiating to glucagon producing α-cells. DNP was used to give stress and it has been shown to enhance the differential potential of NIH3T3 cells by significantly increasing the endocrine marker (Ngn3). A high O_2 condition during the early stage of differentiation is reported to increase the percentage of Ngn3-expressing endocrine progenitor and insulin positive cells in both mESC and hiPSC at the terminus of differentiation via HIF-1α inhibition and stimulation of Ngn3 gene expression (Hakim *et al*., 2014). However, during hypoxia, HIF-1α expression was enhanced which results in the activation of Notch signalling. Activation of Notch results in the inhibition of β-cell differentiation by inhibiting Ngn3 expression. DNP is also used to give chemical hypoxia to the cells as it depletes ATP production (Jovanović *et al*., 2009). However, DNP treatment is reported to suppress HIF-1α expression and enhance Ngn3 expression, as well as it is reported to improve Nrp1 (Ali *et al*., 2015) and VEGF expression (Shavell *et al*., 2012). This increase in Nrp1 expression or enhanced Ngn3 expression via HIF-1α suppression may have led to the increase in insulin level in the treated NIH3T3 cells.

In our study, the strategies to induce efficient trans-differentiation of NIH3T3 cells into IPCs have shown positive endocrine expression pattern, specifically β-cell specific transcription factors, demonstrating their successful regeneration. The study could further be evaluated for their *in vivo* effect and serve as an improved

and effective cellular therapeutic option for type 1 diabetes.

CONCLUSION

It is concluded that DNP has a potential to induce efficient transdifferentiation of NIH3T3 cells into insulin producing β-cells. This study may offer the possibility of improved regeneration of damaged β-cells from mature cell type to functional insulin producing β-cells that could serve as a future therapeutic approach for diabetes.

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IRB approval and ethical statement

Not applicable as the study involves no animals or human subjects.

Statement of conflict of interest

The authors have declared no conflict of interest.

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