Expression in Murine Teratocarcinoma F9 Cells of Transcription Factors Involved in Pancreas Development

L. O'Driscoll, P. Gammell, and M. Clynes

ABSTRACT

Background. Although it has been established that formation and functional differentiation of the pancreas from embryonic endoderm is associated with activation/inactivation of many genes controlled by specific sets of transcription factors, the role and activation sequence of individual transcription factors has not yet been fully elucidated. This study sought to differentiate a murine teratocarcinoma cell line, F9, to endodermal-like cells and, subsequently; to investigate the effects of regulated expression of transcription factors in pancreas development.

Methods. Following differentiation using retinoic acid and db cAMP (RAC), resulting F9 cells (F9-RAC) were transfected with cDNAs for PDX-1, *ngn3*, *beta2/NeuroD* (*beta2*), and *Nkx2.2*, singly or in combination. Expression of these transcription factors was investigated using RT-PCR and immunofluorescence techniques. RT-PCR analysis was used to assess the subsequent effects of expression of these factors on endogenous genes related to pancreas development.

Results. Regulated differentiation of F9 cells generated endodermal-like cell types. Following transfection, PDX-1, *ngn3*, *beta2*, and *Nkx2.2* were expressed in F9-RAC cells, with their proteins localized mainly in cellular nuclei. Expression of these factors apparently did not affect the endogenous expression of preproinsulin, PDX-1, *beta2*, *Isl1*, *Pax4*, *Pax6*, Sonic hedgehog, and Indian hedgehog.

Conclusion. This study describes the successful transient expression of transcription factors related to pancreas development, following directed differentiation of F9 cells to endoderm-like cells, and shows that treatment of F9 cells with a combination of RAC causes up-regulation of genes relevant to pancreatic development. The lack of further effect of regulated transcription factor expression on these genes may suggest that parietal endoderm-like cells derived from F9 cells is not the optimal lineage from which to develop beta cells. It may be useful to include F9-derived visceral endoderm in future studies.

THE PROBLEM OF LIMITED transplant tissue for treatment of insulin-dependent diabetes could, in principle, be overcome by generating beta cells in vitro from pluripotent stem cells. For this reason, extensive research has recently focused on elucidating the mechanisms that stimulate embryonic development of beta cells with the appropriate expression of genes encoding beta cell proteins. Although the pathway(s) involved in this complex process have not yet been determined, it has been established that the pancreas originates from the endodermal layer of the developing embryo, and a number of transcription factors have been identified that are required for the successful

occurrence of these events.¹ These transcription factors include pancreatic duodenal homeobox 1 (PDX-1; also

From the National Institute for Cellular Biotechnology, Dublin City University, Glasnevin, Dublin, Ireland.

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L. O'Driscoll and P. Gammell contributed equally to this article.

Address reprint requests to Lorraine O'Driscoll, National Institute for Cellular Biotechnology, Dublin City University, Glasnevin, Dublin 9, Ireland. E-mail: Lorraine.ODriscoll@dcu.ie

known as *Ipfl*, *Stfl*, and *Idx1*), *Hb9*, *ngn3*, *beta2/neuroD* (*beta2*), *Isl1*, *Hes1*, *Pax4* and *Pax 6*, *Nkx2.2* and *Nkx6.1*, and *HNF-6*.¹⁻³

Both *in vitro* and *in vivo* studies have shown PDX-1 to be essential for endocrine neogenesis,⁴ occurring as one of the first known molecular signs that part of the foregut is committed to pancreatic fate. PDX-1 expression is maintained at all stages of beta-cell development and in the mature beta cell. In development, PDX-1 is apparently involved both in the regionalization of the primitive gut endoderm and the maturation of the pancreatic beta cells,⁵⁻⁶ and, in adult life, it positively regulates expression of insulin, GLUT-2, and glucokinase. PDX-1 apparently represses glucagon expression in beta cells.⁷ Other critical factors include *ngn-3*, *beta2*, and *Nkx2.2*. *Ngn3* has been proposed to be a likely candidate marker of endocrine progenitor cell(s), due to its transient expression in hormone-negative cells just prior to the appearance of endocrine cells,^{8,9} possibly acting as a genetic switch that specifies the endocrine cell fate in pluripotent pancreatic progenitor cells.¹⁰ Stable transfection of *ngn3* into endodermal cells developed by retinoic acid treatment of F9 cells has been reported to result in insulin production in response to glucose stimulation.¹⁰ Expression of *beta2*, one of the first genes induced by *ngn3* following notch-based entry to endocrine differentiation,^{8,11-13} is detected early in the developing pancreas where it seems to facilitate correct spatial organization of the developing islet. Adenovirus-mediated delivery of *beta2/neuroD* to the liver of streptozocin-treated mice partially reversed diabetes.¹⁴ In the absence of *beta2*, PDX-1-mediated induction of insulin gene expression and subsequent transcription of the insulin gene are impaired.¹⁵⁻¹⁸ *Nkx2.2*, a homeobox transcription factor apparently required for terminal differentiation of beta cells, may also be critically required for the expression of the insulin gene. Mice lacking *Nkx2.2* do not develop insulin-producing cells and have reduced numbers of alpha and PP cells. "Presumptive" beta cells, formed in these mice, do not express insulin, but do express other markers of beta cells.¹⁹ Whereas the expression of some transcription factors may be required throughout beta-cell development, studies of mouse models suggest that transient expression of other factors may be adequate.

F9, a murine teratocarcinoma cell line,^{20,21} has been shown to differentiate in the presence of retinoic acid (RA) or a combination of RA and dibutyl cyclic adenosine monophosphate (db cAMP) to endodermal cell types.^{10,22,23} In this study, following induction of F9 differentiation to an endodermal-like cell type, the effects of transient expression of a range of transcription factors associated with pancreas development on expression of relevant endogenous genes were investigated. Cotransfections of transcription factors were included where it was suspected (based on the emerging understanding of pancreatic development) that cooperative effects may result.

MATERIALS AND METHODS

Cell Line Culture

F9 cells (ATCC CRL-1720) were grown on tissue culture vessels coated in 0.1% gelatin (Sigma; G1890) in DMEM containing 4 mmol/L L-glutamine and 10% FCS and were cultured at 37°C with 5% CO₂. Routine sterility checks, including screening for *Mycoplasma*, indicated that the cells were clear of contamination. Differentiation and transient transfection experiments were carried out in six-well plates. F9 cells were seeded at 1 × 10⁴ cells/well, allowed to attach overnight, and were subsequently treated with 10⁻⁷ mmol/L all-trans retinoic acid (RA) (Sigma; R2625) and 10⁻³ mmol/L dibutyl cAMP (db cAMP) (Sigma; D0260) for 72 hours at 37°C in a 5% CO₂ atmosphere, in darkness. The RA + db cAMP cells were subsequently termed "F9-RAC."

Transcription Factor Plasmid Constructs

Murine PDX1 cDNA (pcDNA3-PDX-1) was received in pcDNA3 plasmid as a gift from Y. Kajimoto (Japan); *ngn3* (pcDNA3-*Ngn3*) was kindly supplied in pcDNA3 plasmid as a gift from I. Kojima (Japan); *beta2* cDNA (PCR3.1-Beta2) was received as a gift from J. Tsai (Baylor University, Texas, USA); and *Nkx2.2* was received in PBAT12.sh vector from M. German (UCSF, San Francisco, Calif, USA).

Transient Transfection

Allowing 2 µg plasmid DNA per well, DNA to be transfected was mixed with liposome transfection agent Fugene6 (Boehringer Mannheim, 1 814 443) in a 3:1 ratio, following Fugene6 manufacturer's instructions. In brief, DNA and Fugene6 were mixed in 100-µL serum-free medium (per well to be transfected) and were incubated for 25 minutes at room temperature. During this period, medium was changed on the wells to be transfected and on the control wells. Transfection mix (100 µL) was added to the relevant wells in a drop wise manner, with constant swirling. The transfections were carried out over 48 hours and the cells were subsequently analyzed by RT-PCR and immunofluorescence techniques.

RT-PCR

Total RNA was isolated from cells by extracting with TriReagent (Sigma; Poole, England) according to the manufacturer's instructions. First-strand cDNA was synthesised from 1 µg RNA using oligo dT primers (Oswel; Southampton, England). cDNA (5 µL) was then amplified in a 50-µL PCR reaction solution containing 1.5 mmol/L MgCl₂, 0.2 mmol/L deoxynucleotide triphosphates, 20 µmol/L oligonucleotide primers, and 2.5 U *Taq* polymerase enzyme (Sigma; D4545). Forward and reverse primers for amplification of cDNAs of interest and the amplified product sizes are detailed in Table I. GAPDH was coamplified in all cases as endogenous control. The PCR cycle used was as follows: 94°C for 2 minutes; 30 cycles of 94°C for 30 seconds, relevant annealing temperature (Table 1) for 30 seconds, 72°C for 30 seconds; completion step of 72°C for 5 minutes.

Immunofluorescence

To visualize the nuclear transcription factors PDX-1, *ngn3*, *beta2*, and *Nkx2.2*, cells were fixed in ice-cold methanol at -20°C for 7 minutes, preincubated in 50 mmol/L NH₄Cl (Riedel de Haen; 31107) for 30 minutes, and subsequently incubated in 1 × TBS for 5 minutes, followed by incubation in blocking buffer (5% swine

Table 1. Primers to Amplify cDNA Formed by Reverse Transcription of mRNA Templates

cDNA	Primer sequences	Annealing temperature	Amplified product (bp)
PPI	(F): 5' agcgtggctcttctacacacc 3' (R): 5' ggtgcagcactgatccacaatg 3'	54	158
PDX-1	(F): 5' ggtggagctggcagtgatgtt 3' (R): 5' accgccccactcgggtcccg 3'	60	125
Ngn3	(F): 5' tggcgctcatcccttgatg 3' (R): 5' cagtcacccactctgcttcg 3'	54	160
Beta2	(F): 5' ctggccaagaactacatctgg 3' (R): 5' ggagtaggatgcacccggaa 3'	60	229
Nkx2.2	(F): 5' cggagaaaggtatggagtgac 3' (R): 5' ctggcgctgtactgcatgtgctg 3'	60	188
Isl1	(F): 5' acgtctgattccctgtgtgtgg 3' (R): 5' tcgatgtgttacaccttagagcgg 3'	60	276
Pax4	(F): 5' tggcttctgtccttctgtgagg 3' (R): 5' tccaagacacctgtgcggtagtag 3'	52	242
Pax6	(F): 5' aagagtggcgactccagaagt 3' (R): 5' accatactgtattctgttcagg 3'	60	545
Ihh	(F): 5' aaggcccacgtgcattgctct 3' (R): 5' tgtccgcaatgaagagcaggtg 3'	60	298
GAPDH	(F): 5' catgaccacagtccatgccatc 3' (R): 5' caccctgtgtgtgtagccgtattc 3'	AR	451

Note: AR = as relevant, ie, as GAPDH, was coamplified with other cDNAs as endogenous control, annealing temperature used was as relevant for cDNA of interest.

serum: [Dako; X0901] diluted in PBS-1% BSA) for 1 hour. The primary antibodies—PDX-1 (gift from CVE Wright, Vanderbilt, US; 1/500 dilution), ngn3 (Chemicon AB5684; dilution 1/25), beta2 (Chemicon AB5686; 1/25), and Nkx2.2 (Developmental Hybridoma Bank 74.5A5; neat serum)—were diluted, as relevant, in PBS + 1% BSA + 0.1% Triton X-100. Antibodies were added and allowed to incubate overnight at 4°C in a moist environment. Negative controls were performed by omission of the primary antibody. Cells were washed three times with TBS-0.1% Tween and subsequently labeled secondary antibodies (TRITC-labeled goat anti-rabbit IgG [Dako R0156; 1/40 dilution] in the case of PDX-1, beta2, and ngn3 and FITC-labeled rabbit antimouse IgG [Dako F0261; 1/30 dilution] for Nkx2.2 detection) were applied and incubated at room temperature for 1 hour in darkness. Following this, the cells were washed three times in TBS-0.1% Tween, mounted using fluorescence mounting medium (Dako, S3023), and assessed by fluorescence microscopy.

RESULTS

RT-PCR Analysis of Transiently Expressed Transcription Factors

Following differentiation with all-trans RA + db cAMP and subsequent transient transfection of transcription factors, RT-PCR analysis revealed the overexpression of PDX-1, beta2, ngn3, and Nkx2.2 in F9-RAC cells (Fig. 1a to 1d), indicating successful transfection. It was observed that untransfected F9 cells had low-level expression of each of these gene transcripts and that the levels, prior to transfection, were largely unaffected by treatment with RA and db cAMP. Similarly, cotransfection of transcription factors in

combination apparently did not further increase levels of the transcription factors investigated.

Immunofluorescence Analysis of Transiently Expressed Transcription Factors

Following transient transfection, immunofluorescence analysis indicated that PDX-1 was expressed in the nuclei of F9-RAC cells, with low-level expression apparent in cytoplasmic regions of the cells. Similar staining patterns were found with the control cell line, MIN-6 (Fig 2(a)). Only faint, nonspecific staining occurred in nontransfected F9-RAC cells. Similarly, beta2 expression was clearly present in populations of F9-RAC cells and in the MIN-6 cells, with strong nuclear staining and less intense cytoplasmic staining (Fig 2(b)). Ngn3 and Nkx2.2 were also detected in the nuclei of their respective transfected populations of F9-RAC cells, although the intensity of staining was slightly less than that found for PDX-1 and beta2 (results not shown).

Expression of Endogenous Genes

Endogenous expression of preproinsulin (PPI), PDX-1, beta2, Isl1, Pax6, and Indian hedgehog (Ihh) gene transcripts was detected in untreated F9 cells (Pax4 and sonic hedgehog [Shh] were undetected in all of the F9 populations analyzed). Treatment of F9 cells with RA and db cAMP resulted in increased levels of PPI, PDX-1, Isl1, Pax6, and Ihh transcripts, whereas beta2 levels were apparently unaffected by this treatment. Subsequent overexpression of pancreatic transcription factors, PDX-1, ngn3, beta2, and Nkx2.2 (singly or in combination) apparently did not greatly affect the transcript levels of any of the endogenous genes analyzed (Fig. 3a to 3f).

DISCUSSION

Regulated development of beta cells from pluripotent stem cells in vitro may alleviate the current problem of insufficient transplant tissue for treatment of insulin-dependent diabetes. Pancreatic development is, however, highly complex. Although many studies (mainly on animal models) have reported the molecular events accompanying pancreatic differentiation and development,^{7-10,24} a definitive understanding of the hierarchy of the molecular events is lacking. Conflicting results from studies of animal models may have arisen due to the heterogeneous nature of the cells involved, the undetermined influence of environmental factors, and the coexpression in the cells, at any given time, of many undefined transcription factors. For stem cell differentiation to beta cells to become a routine approach for generation of transplantable cells, an understanding of the order of these events and their subsequent effects on pancreatic gene expression is necessary.

Ectopic expression of ngn3 in RA-derived endoderm F9 cells (following 4-day treatment with RA) has recently been reported to induce the expression of several pancreatic transcription factors in a well-defined sequence; with stable transfectants producing insulin in response to glucose.¹⁰

Fig 1. RT-PCR analysis of F9 and F9-RAC cells demonstrated overexpression of (a) PDX-1, following the transfection of the pcDNA3-PDX-1 plasmid. Overexpression, at a similar level, was also achieved following coexpression of the PDX-1 cDNA with Beta2 and Ngn3, (b) beta2, following transfection of PCR3.1-Beta2. Overexpression was also achieved following cotransfection with the PDX-1 cDNA (pcDNA3-PDX1), (c) ngn3, following transfection of pcDNA3-Ngn3. Overexpression was also achieved following coexpression of the Ngn3 cDNA with the PDX1 cDNA, and (d) Nkx2.2, following transfection of the Nkx2.2 expression vector. H₂O and the murine beta cell line, MIN-6, were used as negative and positive controls, respectively. GAPDH was used as an endogenous control for RNA quality and quantity. The results presented are representative of repeat gels from independent experiments.

Fig 2. (a) **A** PDX-1 and (b) **A** beta2 expression in F9-RAC-PDX-1. MIN-6 cells were used as a positive control (a and b [**C**]). Also included are the relevant phase contrast images (**B** and **D**). Magnification = 40 $\times$.

Beta2 and Pax6 gene transcripts were induced 48 hours after *ngn3* transfection and *Isl1* mRNA was detected after 72 hours, suggesting the transcriptional activation sequence to be *ngn3* as the first event in the temporal sequence of transcription factor activation, followed by coexpression of

beta2 and Pax6, and subsequently *Isl1* expression. Using the same cell line (ie, F9) and differentiating with RA and db cAMP, in the study reported here transient transfection resulted in successful overexpression of PDX-1, beta2, *ngn3*, and *Nkx2.2* in cellular nuclei. However, although the

Fig 3. Endogenous PPI (a), PDX-1 (b), beta2 (c), Isl1 (d), Pax6 (e), and Ihh (f) expression in F9-RAC cells transiently expressing pancreatic transcription factors. F9 and F9-RAC cells are shown for comparison. MIN-6 and water were used as positive and negative controls, respectively. The results shown are representative of repeat gels from independent experiments.

differentiation treatment resulted in increased levels of PPI, PDX-1, Isl1, Pax6, and Ihh gene transcripts, overexpression of PDX-1, beta2, ngn3, and Nkx2.2 transcription factors did not result in further up-regulation of these mRNAs.

The lack of induced expression of pancreatic mRNAs (including beta2, Pax6, and Isl1) following expression of transcription factors described here conflicts with studies reported by Vetere et al.¹⁰ However, there have been a

(d)

(e)

(f)

Fig 3. Continued

number of reported cases where transcription factor expression did not result in the anticipated knock-on effects; for example, nuclear expression of PDX-1 had no effects on insulin expression in α TC1.9 cells,¹⁵ while expression of PDX-1, Isl1, and Nkx6.1 (singly or in combination) had no

detectable effects on expression of genes investigated in AR42J-B13 cells.²⁵ In contrast, expression of PDX-1 in α TC1.6 resulted in induced expression of islet amyloid polypeptide²⁶; in Trm6 cells it induced somatostatin²⁷; in IEC-1 cells it induced glucokinase and Nkx6.1²⁸, and in

Capan 1 cells it resulted in induced expression of insulin,²⁹ while transfection of *ngn3*³⁰ and *Nkx2.2*²⁵ into AR42J cells induced PP expression.

In conclusion, it appears that overexpression of transcription factors may elicit a large affect on one cell type, but not in another; this presumably relates to the preexisting cell phenotype in terms of chromatin structure, expression of transcription factors, etc. In the study reported here it is possible that the parietal endodermal nature (following RA and db cAMP) of the transfected population may be an unsuitable lineage from which to induce pancreas-related genes (although the RA and db cAMP treatment induced expression of relevant genes). Future studies investigating the affects of PDX-1, beta2, and *Isl1* expression in RA-derived endoderm-like cells may assist in determining the affects of such transcription factors in a relevant cell-line model and so may help to identify ways of developing replacement pancreatic beta cells.

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