Rapid Publication

Retinoic Acid Induces *Pdx1*-Positive Endoderm in Differentiating Mouse Embryonic Stem Cells

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We have generated an embryonic stem (ES) cell line in which sequences encoding green fluorescent protein (GFP) were targeted to the locus of the pancreatic-duodenal homeobox gene (*Pdx1*). Analysis of chimeric embryos derived from blastocyst injection of *Pdx1*$^{GFP/w}$ ES cells demonstrated that the pattern of GFP expression was consistent with that reported for the endogenous *Pdx1* gene. By monitoring GFP expression during the course of ES cell differentiation, we have shown that retinoic acid (RA) can regulate the commitment of ES cells to form *Pdx1*$^{+}$ pancreatic endoderm. RA was most effective at inducing *Pdx1* expression when added to cultures at day 4 of ES differentiation, a period corresponding to the end of gastrulation in the embryo. RT-PCR analysis showed that *Pdx1*-positive cells from day 8 cultures expressed the early endoderm markers *Ptf1a*, *Foxa2*, *Hnf4α*, *Hnf1β*, and *Hnf6*, consistent with the notion that they corresponded to the early pancreatic endoderm present in the embryonic day 9.5 mouse embryo. These results demonstrate the utility of *Pdx1*$^{GFP/w}$ ES cells as a tool for monitoring the effects of factors that influence pancreatic differentiation from ES cells. *Diabetes* 54:301–305, 2005

Insulin-producing cells generated from in vitro–differentiated embryonic stem (ES) cells have been advanced as a potential alternative to cadaveric-derived pancreatic islets in transplantation therapies for treatment of type 1 diabetes. The development of protocols that facilitate the reliable and efficient derivation of such cells has been the focus of several studies over the last 5 years. Soria et al. (1) used a "cell-trapping" protocol to select for insulin-producing cells expressing the *Neo*^{R} gene under the control of the human insulin gene promoter. This strategy was refined by placing the *Neo*^{R} gene under the control of the promoter of *Nkx*6.1 (2), a gene found to be important in the development of cells from endocrine precursors. Taking into account the close evolutionary and developmental relationship between endocrine and neural cell lineages, Lumelsky et al. (3) developed a five-step protocol based on methods known to promote the generation of neural cell types from ES cells. Although the nature of insulin-staining cells derived by this method remains controversial (4,5), other groups have successfully used variations on this procedure to isolate similar cells from differentiating ES cells (6,7). Enforced expression of transcription factors with a role in pancreatic development has also been used to increase the frequency with which insulin-producing cells were isolated from differentiating ES cells (8,9).

We have taken an alternative approach to optimize the efficiency of the intermediate stages traversed by ES cells differentiating toward the pancreatic lineages. Pancreatic endocrine cells originate from definitive endoderm that expresses the pancreatic-duodenal homeobox gene (*Pdx1*) (10,11). In the absence of *Pdx1*, the pancreas fails to develop beyond the formation of ventral and dorsal buds (12,13). Thus, *Pdx1* expression marks a critical step in pancreatic organogenesis, and *Pdx1*$^{+}$ cells are likely to represent an obligate intermediate population in the generation of β-cells from ES cells. Therefore, we generated a reporter cell line by inserting the gene encoding green fluorescent protein (GFP) into exon 1 of the *Pdx1* gene to facilitate the optimization of ES cell differentiation toward the pancreatic lineage. Using this cell line, we now show that retinoic acid (RA) promotes the generation of *Pdx1*$^{+}$ cells that express a repertoire of genes indicative of early foregut endoderm.

RESEARCH DESIGN AND METHODS

Construction of targeted ES cells. The *Pdx1*-GFP targeting vector comprised a 2.8-kb DNA fragment encompassing sequences upstream of the *Pdx1* initiation codon, positioned 5′ of a cassette encoding GFP and a hygromycin resistance gene (Hygro^{R}) flanked by flp recombinase target sites. The 3.3-kb 3′ arm of the targeting vector encompassed sequences from an *Mlu*I site in exon 1 to an *Xba*I site immediately 5′ of exon 2. The targeting vector was electroporated into W9.5 ES cells, and targeted clones were identified by a PCR-based approach. Correctly targeted ES cells were transiently transfected with a vector encoding flp recombinase, and a clone of normal karyotype in which the Hygro^{R} cassette had been excised was characterized by Southern blot analysis.

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EB, embryoid body; E-cad, E-cadherin; ES, embryonic stem; GFP, green fluorescent protein; RA, retinoic acid.

TABLE 1
Primers used for PCR analysis

Gene	Sense primer	Antisense primer	Product size (bp)
HPRT	gctggtgaaaaggacctct	cacaggactagaacacctgc	249
E-cad	gcagtcagatctccctgagttcgag	gttgctagagtgacctttgtatgtag	372
Pdx1	ctatccttcaacctataccatttc	gaaatcagccaggttgccttcaac	409
Ptf1a	catagagaacgaaccacccttttgag	gcacggagtttcctggacagagttc	294
Hnf6	gcaatggaagtaattcagggcag	catgaagaagttgctgacagtgc	471
Hnf4α	ctcttctgattataagctgaggatg	ccacaggaaggtgcagattgatctg	377
Foxa2	cctctatgtagactactgcttctc	cctggatttcaccatgtccagaatg	277
Hnf1β	gttgaaattccaagagtgacttgctc	ctttaatgggaggcttcctgagatg	281
Hlxb9	caagctcaacaagtacctgtctcg	gcaccattgctgtacgggaagttg	341
NeuroD	cttggccaagaactacatctgg	ggagtagggatgcaccgggaa	222
Ngn3	ggtagcactacctagttggagactc	gacaaacagtgcttcaggaaccgtc	389
Nkx2.2	ctaaatatttatggccatgtacacg	gttccaagctccgatgctcaggag	325
Insulin1	ccagctataatcagagacca	gtgtagaagaagccacgct	197
Glucagon	actcacagggcacattcacc	ccagttgatgaagtccctgg	353

Chimera analysis. Chimeric embryos generated by injecting *Pdx1*$^{GFP/w}$ ES cells into C57BL/6 blastocysts were harvested at 7 and 10 days' postimplantation and fixed in 4% paraformaldehyde on ice for 5 min. Images of embryos expressing GFP were captured with a Leica fluorescence microscope. This work involving animals was conducted in accordance with Monash University guidelines.

Cell culture. ES cells were maintained on primary mouse embryonic fibroblasts in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum and 10^3 units/ml leukemia inhibitory factor (LIF). For differentiations, feeder-depleted ES cells were seeded at 10,000 cells/ml in 5 ml differentiation medium (14) in 6-cm Petri dishes (Phoenix Biomedical). For RA treatments, embryoid bodies (EBs) were harvested, washed once in PBS, and returned to Petri dishes in chemically defined medium (CDM) (15) supplemented with all-*trans* RA (2625; Sigma). The following day EBs were washed in PBS and a single EB picked into each well of a gelatin-coated 96-well tissue culture plate in CDM. Each EB was subsequently scored for GFP expression using a Zeiss Axiovert fluorescence microscope.

Gene expression analysis. ES cells differentiated for 8 days were stained with an anti–E-cadherin (E-cad) antibody (13-1900; Chemicon), and GFP$^+$E-cad$^+$, GFP$^-$E-cad$^+$, and GFP$^-$E-cad$^-$ cells were isolated by flow cytometry using a FACSAria (BD Biosciences). cDNA was generated using a Cells-to-cDNA II (Ambion) kit and samples standardized essentially as described (16). For PCR analysis, the primer sequences and product sizes are listed in Table 1. Following an initial denaturation step of 95°C (2 min), PCRs were performed for 33 cycles with conditions of 95°C (30 s), 55° (30 s), and 72° (60 s) using High Fidelity Platinum *Taq* polymerase in the presence of 25 mmol/l $MgSO_4$ and 200 μmol/l dNTPs in the buffer supplied (Invitrogen). PCR products were separated by electrophoresis on a 2% agarose gel.

RESULTS

Sequences encoding GFP were inserted into exon 1 of the *Pdx1* locus in mouse ES cells using homologous recombination (Fig. 1*A*), and correct targeting was verified by Southern blotting (Fig. 1*B*). We examined the pattern of GFP expression in chimeric embryos derived by blastocyst injection of *Pdx1*$^{GFP/w}$ ES cells. Embryos that recovered at day 7 postimplantation (developmentally equivalent to embryonic day [E] 9.5) showed two areas of GFP expression (Fig. 1*C*) associated with the forming gut tube. This pattern of expression in prospective dorsal and ventral pancreatic buds was identical to that reported for the endogenous *Pdx1* gene (17,18). Robust GFP fluorescence was observed in the dorsal and ventral pancreatic anlage, and lower levels were present in the duodenum of day 10 (developmentally E12.5) postimplantation embryos (Fig. 1*D*). GFP expression was not detected in other embryonic tissues.

Studies in zebrafish and *Xenopus* showed that the proportion of cells allocated to pancreatic endoderm could be increased by treating embryos with RA toward the end of gastrulation (19,20). Preliminary studies in our laboratory indicated that a 24-h pulse with RA was also able to induce GFP expression in differentiating *Pdx1*$^{GFP/w}$ ES cells and that continuous presence of RA was not required (data not shown). To determine the optimal concentration of RA in our system, ES cells differentiated for 4 days were treated with various concentrations of RA for 24 h and observed for subsequent GFP expression (Fig. 2*A*). The highest proportion of GFP$^+$ EBs (>90%) formed when cultures were treated with 10^{-5} mol/l RA. This number peaked 3–4 days following RA treatment and decreased gradually over the next 7 days. To determine whether the time of treatment influenced the frequency of GFP$^+$ EBs, cultures were pulsed with RA for 24 h between days 2 and 10 of differentiation. These experiments indicated that exposure of EBs to RA at day 4 yielded the highest percentage of GFP$^+$ EBs (Fig. 2*B*). Examination of day 8 EBs from these cultures revealed that GFP expression was localized to epithelial structures (Fig. 2*C*), often in close proximity to cardiac mesoderm (data not shown).

Flow cytometric analysis indicated that GFP$^+$ cells present in day 8 EBs treated with RA at day 4 coexpressed the epithelial marker E-cad (Fig. 3*A*). To determine the developmental stage represented by the GFP$^+$ cells, RT-PCR analysis was performed on RNA from cells isolated on the basis of GFP and E-cad expression. The purity of the sorted populations was verified by RT-PCR analysis, showing that cells expressing *Pdx1* and *E-cad* RNA were confined to GFP$^+$ and E-cad$^+$ fractions, respectively (Fig. 3*B*). This analysis indicated that the GFP$^+$ population expressed endoderm markers including *Foxa2*, *Hnf4α*, *Hnf6*, and *Hnf1β*. Although these cells did not express genes associated with later pancreatic differentiation, the expression of *Ptf1a* suggests that a proportion of the GFP$^+$ population was committed to pancreatic endoderm (21) (Fig. 3*B*).

DISCUSSION

The efficient differentiation of ES cells into β-cells will require the optimization of a series of steps corresponding to the sequential stages of pancreatic development (11). To facilitate the isolation of pancreatic endoderm from

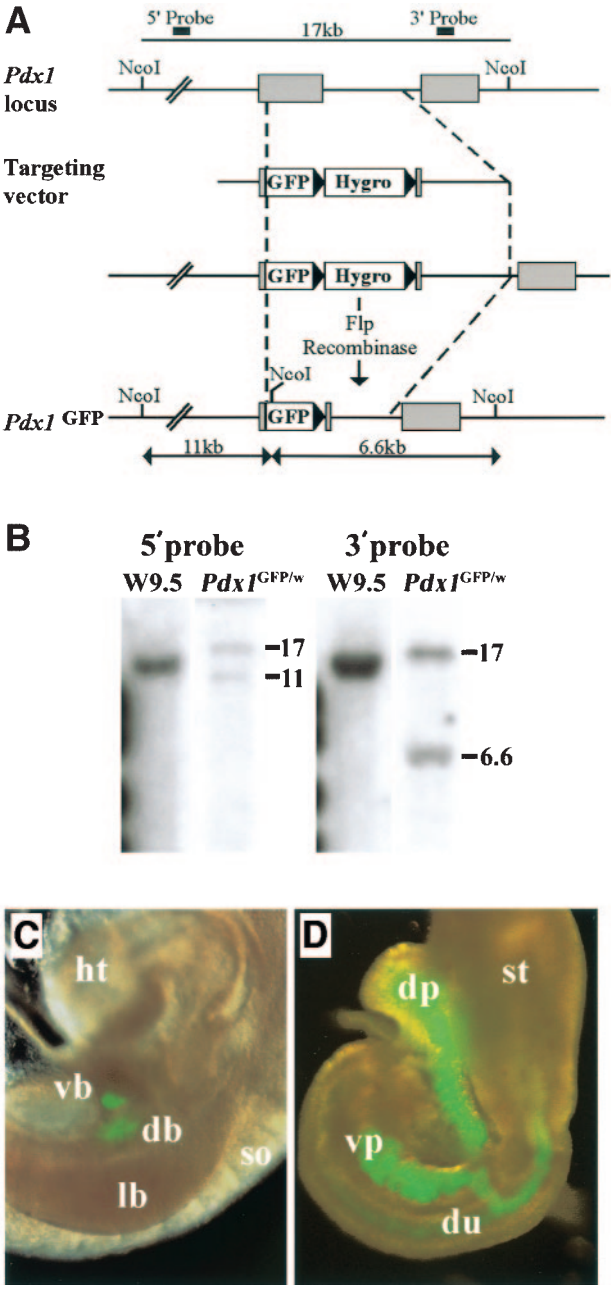

FIG. 1. Generation of *Pdx1*$^{GFP/w}$ ES cells. *A*: Schematic representation of the gene-targeting vector used to insert GFP into the endogenous *Pdx1* locus by homologous recombination. 5′ and 3′ probes located outside the targeting vector detect a 17-kb *Nco*I fragment in the wild-type *Pdx1* allele. This fragment is disrupted in the targeted allele by the presence of an additional *Nco*I site in GFP. Gray boxes denote exons, and black triangles represent flp recombinase target sites flanking the hygromycin resistance cassette (Hygro). *B*: Southern blot of *Nco*I-digested genomic DNA from wild-type and *Pdx1*$^{GFP/w}$ ES cells showing that 5′ and 3′ probes detect fragments of the predicted size (kb). *C* and *D*: Images of chimeric embryos generated from blastocyst injection of the *Pdx1*$^{GFP/w}$ ES cells recovered 7 (*C*) and 10 (*D*) days postimplantation. The embryo in *D* has been dissected to show the foregut and developing pancreas. lb, limb bud; ht, heart; so, somites; db, dorsal pancreatic bud; vb ventral pancreatic bud; st, stomach; du, duodenum; dp and vp, dorsal and ventral pancreatic anlage, respectivley.

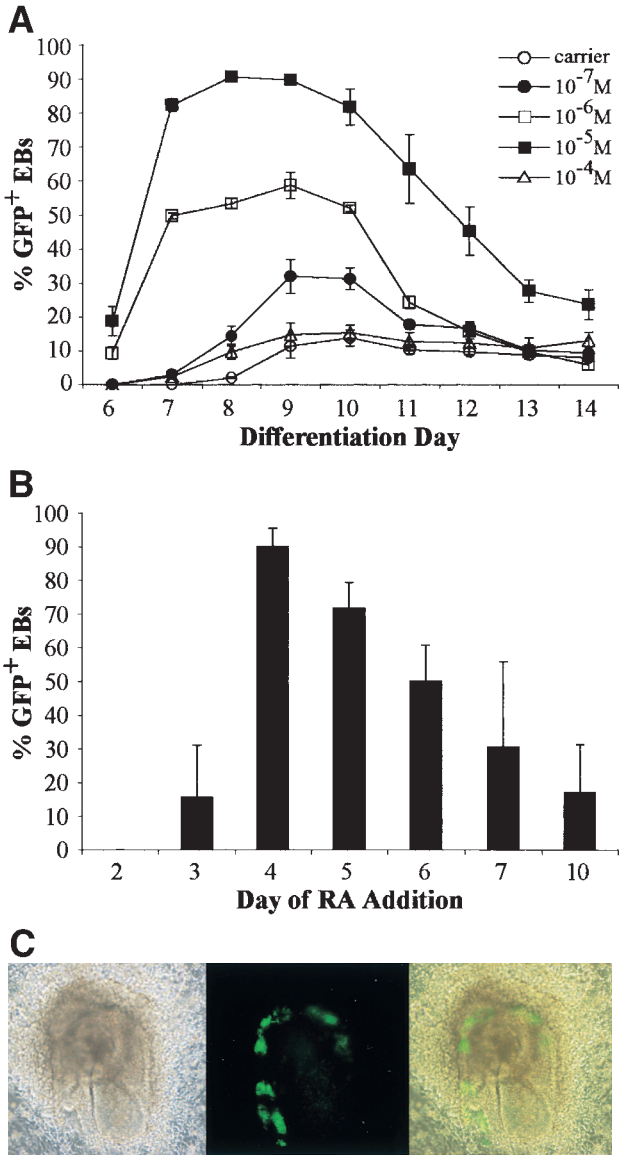

FIG. 2. RA induces GFP expression in differentiating Pdx1$^{GFP/w}$ ES cells. *A*: GFP expression (%GFP$^+$ EBs) as a function of time (differentiation day) following treatment of day 4 EBs with RA or carrier (DMSO) at the concentrations indicated (values shown are means ± SE, n = 3). *B*: Frequency of GFP-expressing EBs 3 days after RA treatment at the days indicated (values shown are means ± SE, n = 3). *C*: Bright field (*left*), fluorescence (*center*), and merged images of a typical day 8 EB (treated with RA at day 4) showing areas of GFP expression localized to epithelial structures (*right*).

differentiating ES cells, we used gene targeting to insert the gene encoding GFP into the *Pdx1* locus. Analysis of chimeric embryos generated with *Pdx1*$^{GFP/w}$ ES cells showed that the pattern of GFP expression mirrored that previously reported for *Pdx1*, and GFP$^+$ cells isolated by flow cytometry expressed *Pdx1* RNA. These data led us to conclude that GFP expression faithfully reported expression of the endogenous *Pdx1* gene and therefore provided a reliable marker of cells within the pancreatic endoderm differentiation pathway. Consistent with findings of studies in zebrafish and *Xenopus* (19,20), our experiments show that *Pdx1* expression was induced in differentiating ES cells treated with RA. Analysis of these *Pdx1*$^+$ cells shows that they expressed a suite of transcription factor genes diagnostic of early foregut endoderm present in the mouse embryo between E8.5 and E9.5 (11). Time course analysis showed that GFP expression diminished by day 14 of differentiation, suggesting that additional factors were required to guide further pancreatic differentiation.

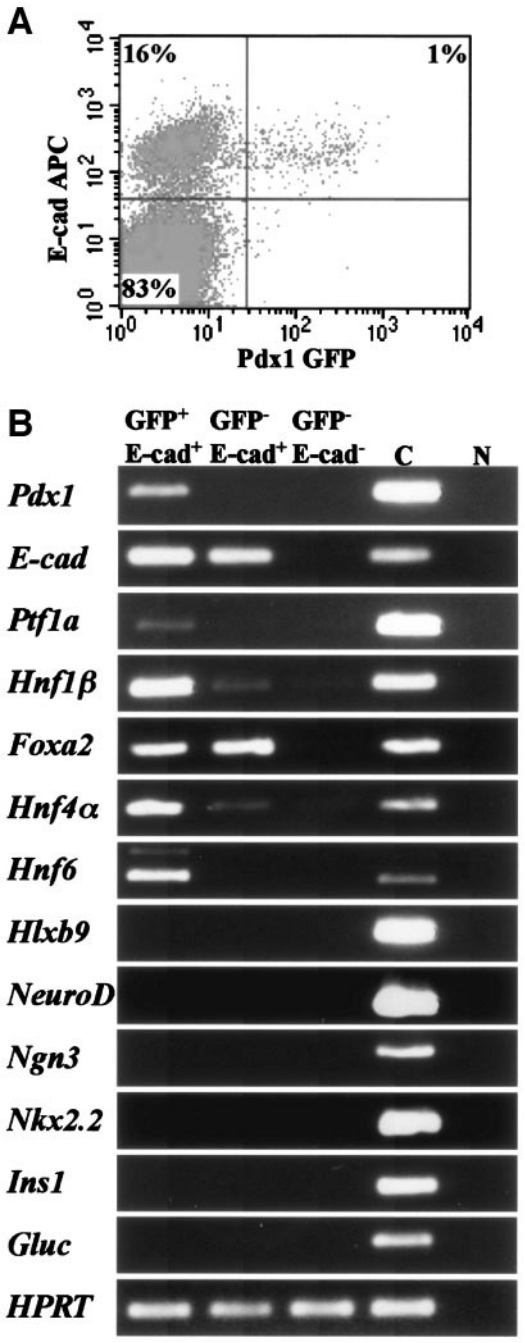

FIG. 3. Day 8 *Pdx1*$^{GFP/w}$ EBs treated with RA at day 4 express markers of early pancreatic endoderm. *A*: Flow cytometric analysis of *Pdx1*$^{GFP/w}$ EB cells showing that all GFP$^+$ cells coexpress E-cad. *B*: RT-PCR analysis of RNA derived from day 8 EB cells sorted on the basis of GFP and E-cad expression. C, control RNA derived from Min6 cells (25), fetal or adult pancreas; N, no template.

However, it is also possible that endogenous factors produced in our cultures actively repressed continuing pancreatic development. Experiments by Deutsch et al. (22) showed that ventral foregut endoderm adopted a default pathway of pancreatic commitment that could be diverted to *Pdx1*$^-$ hepatic endoderm by the proximity of cardiac mesoderm. Their results suggested that mesoderm-derived fibroblast growth factor (FGF) induced the local production of sonic hedgehog (Shh), a factor previously reported to repress *Pdx1* expression in the dorsal foregut endoderm (23). However, although cardiac mesoderm was a prominent feature in our cultures, addition of either FGF2 or inhibitors of Shh signaling to day 8 GFP$^+$ EBs did not modulate subsequent GFP expression (data not shown).

Our experiments show that RA can promote the formation of *Pdx1*$^+$ foregut endoderm that coexpresses *Ptf1a*, a transcription factor indicative of pancreatic commitment (21). However, the absence of markers of further pancreatic differentiation, such as *Ngn3*, *NeuroD*, *Nkx2.2*, and *Insulin*, emphasize that these experiments describe only the first step in the development of a protocol for the differentiation of ES cells into pancreatic β-cells. Indeed, studies by Mandel et al. (24) demonstrated that pancreatic tissue from E12 fetal mice required 2 weeks of maturation in vitro before it could contribute to the regulation of blood glucose levels when transplanted into animals. In this context, the *Pdx1*$^+$ cells characterized in this study are also likely to require further culture before they reach a developmental stage capable of regulating glucose levels in vivo. The definition of culture conditions that will facilitate such further development will form the basis of future work.

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