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Viral-Mediated Coexpression of Pdx1 and p48 Regulates Exocrine Pancreatic Differentiation in Mouse ES Cells

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ABSTRACT

Embryonic stem cells (ES) can spontaneously activate a pancreatic differentiation program *in vitro*, although with low efficiency. The aim was to improve such process by using viral mediated gene transduction. In this study, we have examined the suitability of using viral vectors to express key transcriptional factors involved in pancreatic development. ES cell lines that constitutively express Pdx1, a homeodomain protein involved in both exocrine and endocrine pancreatic development and differentiation, were established using a lentiviral vector. These cells were additionally infected with an adenovirus expressing p48, a bHLH factor that is also crucial for pancreatic development and acinar differentiation. Quantitative RT-PCR analysis demonstrated an increase in the expression of exocrine genes, including those coding for both digestive enzymes and transcription factors. Immunocytochemical staining also revealed an increase in the number of amylase-expressing cell clusters. However, other important genes involved in acinar cell maturation (i.e., Mist1) were not modulated under these conditions, suggesting that the cells display features of immature exocrine cells or because of an uncoupled gene expression of the exocrine differentiation program. Importantly, this effect was selective for the acinar lineage as the expression of a large set of endocrine markers remained unchanged. Therefore, combined expression of key genes involved in pancreatic development may be a promising approach to generate mature pancreatic exocrine cells.

INTRODUCTION

PANCREAS DEVELOPMENT is a complex, regulated process involving the integration of signals originating from mesodermal tissues and resulting in the activation of expression of key transcription factors. Pdx1 is a homeodomain protein that is initially detected at E8.5 in the primitive gut that will subsequently give rise to the pancreas. Targeted inactivation of Pdx1 leads to pancreatic agenesis (Jonsson et al., 1994; Offield et al., 1996). When the expression of Pdx1 is abrogated after bud formation, acinar and islet cells fail to differentiate (Holland et al., 2002). Moreover, lineage-tracing experiments have demonstrated that all pancreatic epithelial cell types originate from progenitors that expressed Pdx1 at some point (Gu et al., 2002). In the adult, Pdx1 regulates the expression of both insulin and exocrine digestive enzymes (Liu et al., 2001; Swift et al., 1998). p48 (also known as PTF1a)

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is a basic helix-loop-helix (bHLH) protein initially described to be involved in the regulation of acinar gene expression (Liu et al., 2001; Rose et al., 2001). Subsequently, p48 was found to be essential for the development of both pancreatic components, as *Ptf1a* null mice lack a pancreas, and single cells expressing pancreatic hormones can be detected in the spleen (Krapp et al., 1998). Later, lineage-tracing studies have shown that p48 is expressed at very early stages in the embryonic pancreas and that all pancreatic epithelial cells originate from a p48-expressing progenitor. In the absence of p48, these progenitors assume an intestinal fate (Kawaguchi et al., 2002). Very recently, in the *Xenopus* model, a combination of both Pdx1 and p48 activities has been shown to be sufficient to expand the pancreatic precursor cell population into portions of the more posterior endoderm. This leads to the formation of a giant pancreas that carries both endocrine and exocrine cells. These experiments showed that the coexpression of these two factors is sufficient to convert nonpancreatic endodermal cells into pancreatic precursor cells (Afelik et al., 2006). Therefore, both Pdx1 and p48 transcription factors are essential for pancreatic development/differentiation.

Murine embryonic stem (ES) cells, derived from the inner mass of blastocyst-stage embryos, are pluripotent and capable of differentiation into all somatic cell lineages *in vitro* and *in vivo*. Thus, when induced to form aggregates (embryoid bodies, EB), ES cells can acquire mesoderm and ectodermal-derived phenotypes (Eiges and Benvenisty, 2002; Fuchs and Sagre, 2000; Rathjen and Rathjen, 2001). Recently, protocols developed using human ES cells have shown that they can be also differentiated into endoderm with high efficiency (D'Amour et al., 2005, 2006). As occurs during *in vivo* development, endodermal precursors within EB can specify into both pancreatic endocrine and exocrine lineages in a process that recapitulates many aspects of early embryonic pancreatic development (Assady et al., 2001; Kahan et al., 2003; Skoudy et al., 2004). In addition, insulin-expressing cells have been isolated by genetic selection using a chimeric construct which allows the expression of the βgeo fusion gene under the control of the human insulin or the Nkx6.1 gene promoters (Leon-Quinto et al., 2004; Soria et al., 2000). Lumelsky et al. have developed a five step protocol based on the selection of nestin-expressing cells to generate endocrine beta-like cells, although the origin of the insulin detected in those cells is unclear (Hansson et al., 2004; Lumelsky et al., 2001; Rajagopal et al., 2003; Segev et al., 2004; Sipione et al., 2004). Other studies have taken advantage of cell proliferation inhibitors and specific growth factors to coax ES cell differentiation into insulin-producing cells (Hori et al., 2002; Moritoh et al., 2003). We have previously shown that incubation of EB with conditioned medium (CM) obtained from the culture of fetal pancreatic rudiments leads to an increase in the expression of pancreatic markers (Skoudy et al., 2004). However, in this system even when using the best combinations of pancreatic growth factors identified, the efficiency was far from optimal. Overexpression of single transcription factors has also been explored as an alternative strategy. Thus, transfection of Pax4 cDNA and conditional expression of Pdx1 in ES cells promoted the differentiation of insulin-producing cells with beta-cell features (Blyszczuk et al., 2003; Miyazaki et al., 2004). Recently, induced overexpression of ngn3 in ES cells lead to an increase in the expression of endocrine hormones (Treff et al., 2006). It is reasonable to conceive that expression of multiple specific pancreatic transcription factors as well as the integration of other methods in a single process could help at improving previous approaches.

In this study, we have tested the hypothesis that overexpression of Pdx1 and p48 in differentiating ES cells would favor the acquisition of a pancreatic phenotype. We have taken advantage of the use of lentiviral and adenoviral vectors to efficiently transduce gene expression. In addition, we have used CM derived from the culture of fetal pancreatic rudiments to simultaneously provide differentiating cells with specific signals required for pancreatic development. Our data demonstrate that delivery of multiple transcription factors can be achieved successfully using such an approach. We show that p48 directs selectively the expression of an acinar phenotype in cells expressing Pdx1, an effect that is enhanced under cell culture conditions that favor pancreatic differentiation.

MATERIALS AND METHODS

Cell culture and in vitro *differentiation*

Murine embryonic stem cells (CGR8) were routinely propagated as previously described (Skoudy et al., 2004). To induce differentiation,

cells were allowed to aggregate in 150-mm bacterial dishes at a density of 8.5×10^4 cells/mL in medium without LIF (leukemia inhibitory factor). After 7 days, 50–100 EB were plated in gelatin-coated six-well cell culture dishes. Medium was changed every other day until the end of the culture period (14–21 days). In some experiments, CM obtained from the culture of E16.5 fetal pancreas was added during cell aggregation and after infection with adenoviral vectors at a 1:1 dilution with normal medium (Skoudy et al., 2004).

Viral generation and gene transduction

R4SA-EFS-eGFP-W and *R4SA-EFS-Pdx1-W* were generated from *pSIV-gaMES4* (Mangeot et al., 2002) by replacing the fragment containing a *CMV-GFP* cassette with fragments containing *EFS-eGFP-W* (a gift from Patrick Salmon) or murine Pdx1 cDNA provided by C. Wright. Method for producing SIV-based vectors in 293 cells have been fully described elsewhere (Mangeot et al., 2000). ES cells (1×10^5) were infected for 5 h with 0.5 mL of supernatant, washed in PBS, and cultured for 48 h. The infected population was subcloned by limiting dilution in 96-well plates. Individual clones were amplified and analyzed for expression of Pdx1 by Western blot.

Adenoviral vectors were obtained as follows. Full-length cDNA encoding rat p48 (obtained as described in Rodolosse et al., 2004) was inserted into the pAd–shuttle–CMV vector (He et al., 1998) to generate pAd–shuttle–CMV–p48. pAd–shuttle–CMV–p48 and pAd–shuttle–CMV–lacZ (a gift from Gene Vector Production Network) were used by the Laboratory of Gene Therapy (Gene Vector Production Network, Nantes, France) to produce recombinant adenoviruses using the Ad-Easy system (He et al., 1998). For adenoviral infections, EB were generated and plated on tissue culture dishes for 36 h as described above. After washing three times with PBS, EB were infected for 12 hours with recombinant adenoviruses at a multiplicity of infection (MOI) of 1:25 in serum-free medium. Virus was eliminated by washing; EB were further cultured in medium supplemented with FBS or with conditioned medium. Under these conditions, the viability of the cells was not compromised as scored by trypan blue dye exclusion in replicated wells.

Western blotting using extracts from transduced cells was performed as described elsewhere (Adell et al., 2000).

RT-PCR analysis

RNA was prepared using the GenElute mammalian total RNA kit (Sigma, St. Louis, MO) and treated with DNase I using the DNA-free kit (Ambion, Austin, TX). RNA (0.5 μg) was reverse transcribed in one step according to the manufacturer's instructions (Qiagen, Valencia, CA). PCR was performed for 30–35 cycles, except for β-actin and Hprt (25 cycles), using the previously described PCR conditions and primers (Skoudy et al., 2004). Primers used to detect additional transcripts include: nestin (Berman et al., 2002), rat p48 (S: CTGTCTCGCCTACCCTTGCA and AS: GCCGGCCTGTGAGAGCTT), and syncollin (S: ATGTCCCTGCTGTGCCCACT and AS: TTGCAGTAGAGGGCAGAGAT. RT-PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

Real-time RT-PCR analysis for carboxypeptidase A, p48, Ngn3, Nkx6.1, insulin I, and the control housekeeping gene Hprt was performed using ABI Prism 7900HT Sequence Detection System and the following predeveloped TaqMan assay reagents (Applied Biosystems, Norwalk, CT): Mm00465942_m1 for carboxypeptidase A, Mm00481616_m1 for chymotrypsinogen, Mm00712898_m1 for elastase I, Mm00479622_m1 for p48, Mm00437606_s1 for ngn3, Mm00454962_m1 for nkx6.1, Mm01259683_g1 for insulin I, and Mm00446968_m1 for Hprt. The PCR reaction was performed according to the manufacturer's protocol and the data were processed using SDS 2.1 software using Hprt mRNA levels as control to determine fold regulation of exocrine enzyme transcripts (Applied Biosystems).

Immunocytochemical analysis

Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% TX-100 and 0.1% saponin for 30 min. Endogenous peroxidase activity was blocked with H_2O_2 for 10 min. After incubation with 1% bovine serum albumin (BSA) for 30 min, primary rabbit antibodies raised against Pdx1, p48, and amylase (Sigma) were added for 1 hr. Cells were then washed with phosphate-buffered saline (PBS) and incubated with the Envision secondary reagent (Dako, Carpenteria, CA) for 30 min. The reactions were developed using DAB as a chromogen. Alternatively, immunofluorescence staining was conducted using the Tyramide Signal Amplification method (TSA™ Fluorescence Systems, Perkin

Elmer, Norwalk, CT) following manufacturer's instructions. Nuclear labeling was performed with ToPro-3 iodide (Molecular Probes, Leiden, The Netherlands). Immunofluorescence staining and GFP expression were visualized with a Leica TCS-SP2 confocal microscope. To detect the β-galactosidase activity *in situ*, cells were incubated with a solution containing 2 mM $MgCl_2$, 5 mM K ferricyanide, and 1 mg/mL X-Gal (Sigma) for 12 h at 37°C.

Statistics

Statistical differences were analyzed by the Student *t*-test, and *p* values <0.05 were considered statistically significant.

RESULTS

Generation of mouse ES cell lines stably expressing Pdx1

To enforce Pdx1 expression in CGR8 cells, a lentiviral vector designated Lv Pdx1 was used. Four independent undifferentiated ES cell lines were established that constitutively expressed Pdx1 after infection, detected using western blotting (Fig. 1A) and immunocytochemistry (Fig. 1B). Three of the lines expressed similar levels of the transgene whereas one, designated ES4Pdx1, showed lower expression levels. Because it has been reported that high levels of expression of Pdx1 in ES cells can be toxic and acinar cells express lower levels of Pdx1 than beta-cells, this line was chosen for further assays (Miyazaki et al., 2004; Smart et al., 2005). Nonetheless, no major differences between the different clones were observed in the results obtained in the *in vitro* differentiation experiments (not shown). Control cell lines were generated after infection with a lentiviral vector coding for GFP (Lv GFP) (Fig. 1B). Pdx1 overexpression did not affect cellular morphology or the proliferative ability of CGR8 cells (data not shown).

To confirm that Pdx1 expression was stable, immunocytochemical assays were used. As shown in Figure 1B, expression of both Pdx1 and the control GFP transgene was maintained in undifferentiated cells after culture of ES4Pdx1 or ESGFP lines for several passages in the presence of LIF (Fig. 1B, a and c). EB from these two lines were generated by culturing ES cells in suspension for 7 days in the absence of LIF, then allowing the aggregates to adhere to gelatin-coated cell culture dishes for an additional 14 days (Fig. 2). As shown in Figure 1B, b and d, expression of both Pdx1 and GFP in most cells was observed with heterogeneous expression levels. The expression of the transgenes appeared to not affect the cell growth of the differentiated cells.

Effect of coculture with conditioned medium from pancreatic rudiments on Pdx1-expressing EB cells

We have previously shown that mouse fetal pancreas CM increases the expression of exocrine genes upon ES cell differentiation (Skoudy et al., 2004). To investigate the additional effect of Pdx1 overexpression, ESGFP and ES4Pdx1 cells were differentiated as described above and incubated with CM obtained from the culture of E16.5 fetal pancreases. Pancreatic marker expression was assessed by semiquantitative RT-PCR. Exogenous Pdx1 increased the expression of several exocrine mRNA transcripts encoding digestive enzymes such as chymotrypsinogen, amylase, and elastase (Fig. 3A). The effect was greater when fetal pancreatic CM was added: a significant increase in the percentage of cells immunoreactive for p48 and amylase (4.1-fold and 6.7-fold, respectively; $p < 0.05$) was observed in cultures expressing exogenous Pdx1 and incubated with fetal pancreatic CM, as shown by immunofluorescence staining (Fig. 4). Carboxypeptidase A and nestin (Delacour et al., 2004; Esni et al., 2004) mRNAs were unchanged (Fig. 3A). Importantly, transcripts for glucagon, insulin, or endocrine transcription factors (i.e., Pax6, Ngn3) appeared to be mainly downregulated or remained unchanged (Nkx6.1) when Pdx1 was overexpressed (Fig. 3A). Quantitative RT-PCR for selected exocrine and endocrine markers confirmed these findings, as shown in Figure 3B. In particular, we were unable to detect insulin I mRNA transcripts. Therefore, ectopic expression of Pdx1 enhances the effect of the pancreatic soluble factors on the expression of a large panel of pancreatic genes.

Kinetics of exogenous p48 expression using an adenoviral vector

Expression of Pdx1 superimposed to the fetal pancreatic signals leads to a marked increase in p48 mRNA in differentiating cells (Fig. 3A). To assess the added effect of p48 expression, differentiating ES4Pdx1 cells were infected with ade-

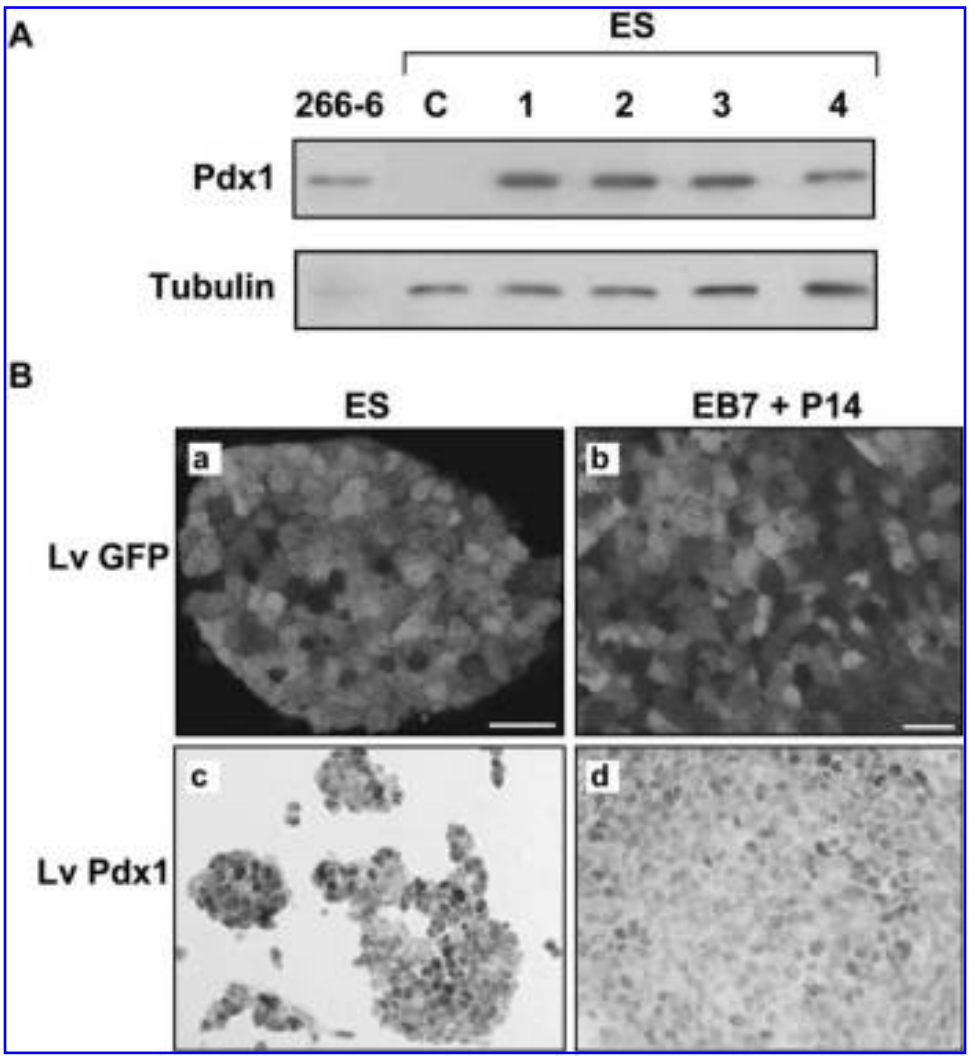

FIG. 1. Generation of ES cell lines stably expressing Pdx1 or GFP using lentiviral vectors. (**A**) Undifferentiated ES cell lines were generated after infection with lentiviruses expressing mouse Pdx1 or GFP. (**A**) Cell extracts of the corresponding cell lines (1–4) were subjected to Western blot analysis using an anti-Pdx1 antibody. Membranes were reprobed with an anti-α-tubulin antibody for protein quantity normalization. Positive control: mouse pancreatic 266–6 cell line. C, control CGR8 cells; ES, undifferentiated mouse ES cells. (**B**) Undifferentiated ES4Pdx1 cells, as well as their differentiated derivatives, were subjected to immnocytochemistry with an anti-Pdx1 antibody. GFP expression in ESGFP cells was analyzed by confocal microscopy. EB7 + P14 refers to the differentiation protocol used as indicated in Figure 2. Original magnification $\times$200. Scale bars = 25 μm.

novirus expressing either LacZ (AdLacZ) cDNA, as a control, or p48 (Adp48) (Fig. 5A). Adenoviral vectors were selected because they allow a more transient expression, thus avoiding a strong antiproliferative effect of p48 (Rodolosse et al., 2004). ES4Pdx1 cells were cultured in suspension for 7 days, and EB were infected with adenoviruses (Fig. 2). Transgene expression levels and cell survival indicated that an MOI of 25 was most favorable: 70% cells were infected with >90% viability at day 2 (Fig. 5B).

Expression of the transgenes was assessed using immunocytochemistry with anti-p48 antibodies and β-galactosidase assays 2–21 days after infection. Figure 5B shows a decrease of p48 and β-galactosidase activity at day 7. At day 21, p48 and LacZ activity were detectable in very few cells. To discriminate exogenous and endogenous p48 mRNA, RT-PCR was conducted using species-specific primers. High levels of rat p48 transcripts were detected 2 and 7 days after infection with Adp48, but not with AdLacZ, which decreased by day 14 (Fig. 5C). This dynamic expression pattern is similar to that observed using immunocytochemical assays. In conclusion, transient ectopic expression of p48 is maintained in

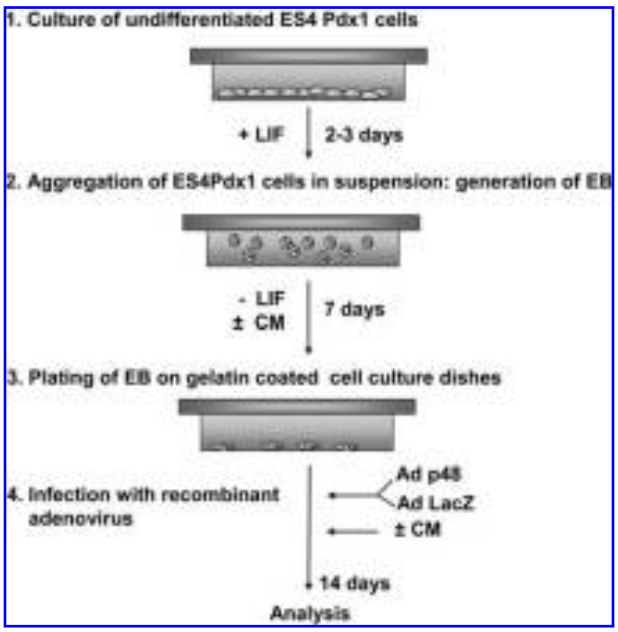

FIG. 2. Schematic representation of the protocol used for *in vitro* differentiation of ES4Pdx1 cells. ES4Pdx1 cells were differentiated in suspension as EB for 7 days, seeded in gelatin-coated cell culture dishes and further grown for 14–21 days. Adenoviral infections were performed in recently adhered EB. In some experiments, CM generated from the culture of E16.5 fetal pancreatic rudiments was added, as indicated.

EB-derived ES4Pdx1 cells for 7 days, providing a time frame for activation of the p48-dependent differentiation program.

Effects of p48 expression on in vitro *differentiation*

To investigate the effect of the simultaneous expression of Pdx1 and p48 in EB incubated with pancreatic CM, semiquantitative RT-PCR was performed in ES4Pdx1 cells infected with adenovirus as described above. Exogenous p48 strongly upregulated amylase and chymotrypsinogen mRNAs, as well as those of carboxypeptidase A (Fig. 6A), a gene whose expression was not modulated in previous experimental conditions (Fig. 3). Transcript levels of Mist1, a bHLH involved in the acquisition and maintenance of the mature exocrine phenotype (Pin et al., 2001), were unaffected. Similarly, p48 expression did not affect the levels of mRNAs coding for endocrine transcription factors (NeuroD, Nkx6.1, Isl1, and Pax6) or for hormones.

Pancreatic marker expression was also analyzed by quantitative RT-PCR (Fig. 6B). Upon p48 gene transduction, carboxypeptidase A and chymotrypsinogen mRNA levels were increased by 14.4- and 12.2-fold, respectively. Exogenous p48 upregulated by 2.4-fold the expression of endogenous p48, suggesting a positive feedback mechanism favoring exocrine differentiation. The lack of regulation of mRNAs for endocrine markers was confirmed (Fig. 6B). In experiments using ESGFP cells, p48 transduction resulted only in a slight increase in exocrine marker expression and endogenous p48 expression was not activated (data not shown); the effects were much higher when Pdx1-expressing cells were used for p48 transduction, as shown by quantitative RT-PCR (Fig. 6C). Furthermore, the combination of transduction with Pdx1 and p48 led to significantly lower levels of the proendocrine gene Ngn3 (Fig. 6C).

Immunocytochemistry was used to analyze acinar marker expression in ESPdx1 cells. As shown in Figure 7A and B, the combination of ex-

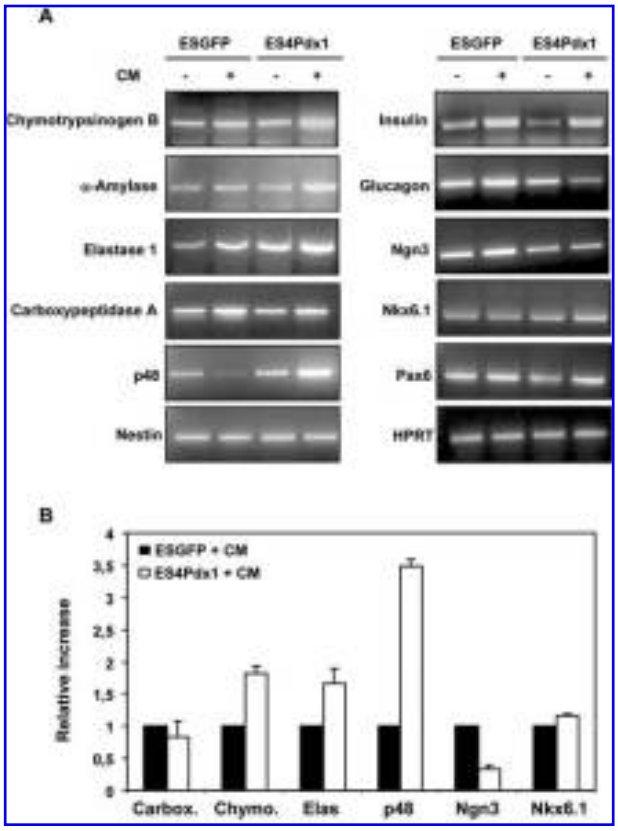

FIG. 3. Effect of Pdx1 on gene expression of EB cultured with fetal pancreas CM. (**A**) Semiquantitative RT-PCR analysis was performed with RNA obtained from differentiated ES4Pdx1 and ESGFP cells cultured with or without fetal pancreatic CM. (**B**) Quantitative RT-PCR analysis for the expression of selected pancreatic markers using the RNA samples obtained in **A.** Histograms show the relative expression levels normalized to the loading control Hptr. Error bars indicate the standard deviations of two experiments performed in triplicate.

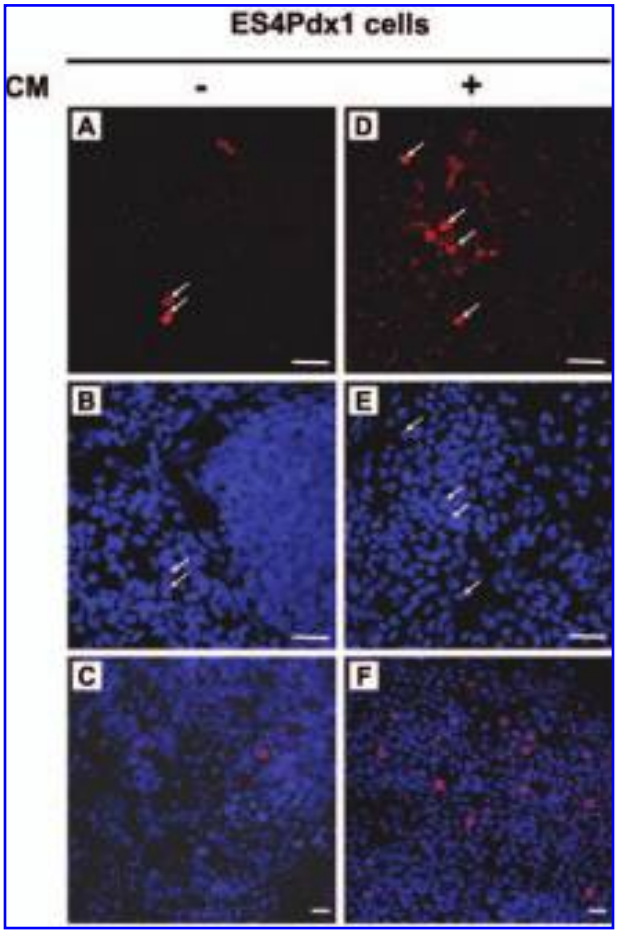

FIG. 4. Immunofluorescence analysis of differentiated ES4Pdx1 cells incubated with or without CM. Staining was performed using specific antibodies for p48 (**A**, **D**), and amylase (**C**, **F**) (red). Nuclei were labeled with ToPro-3 iodide (blue). (**B**, **E**) The nuclei of the cells corresponding to panels **A** and **D**. Arrows in (**A**, **B**, **D**, **E**) show immunoreactive cells. p48 is expressed in the nucleus, whereas amylase displays a cytoplasmic distribution. Scale bars = 25 μm.

ogenous p48 and pancreatic CM led to a marked increase in the percentage of amylase-expressing foci only in Pdx1-expressing cells cultured with CM (g,h vs. a,b vs. e,f): p48 expression in the presence of CM led to a threefold increase in the percentage of foci with strong amylase staining (Fig. 7B). These results demonstrate that transient expression of p48 in Pdx1-expressing cells, in the presence of CM, leads to a selective upregulation of exocrine genes in differentiating ES cells.

DISCUSSION

ES cells can spontaneously differentiate into endodermal lineages, including pancreatic exocrine cells, at low efficiency. The aim of this work was to improve methods to direct acinar pancreatic differentiation of ES cells using viral vectors containing the cDNA of transcription factors to develop *in vitro* differentiation-based strategies to ultimately overcome pancreatic insufficiency, such as in chronic pancreatitis. Our strategy relies on the simultaneous expression of two key transcriptional regulators in differentiating ES cells. Here, we combine the advantages of lentiviral vectors, to ensure constitutive expression of transgenes (Ma et al., 2003; Pfeifer et al., 2002), and adenoviral vectors to allow transient gene expression (Lundstrom, 2003). This strategy allows for the rapid assay of transcription factor combinations in ES cells, and may facilitate to better reproduce the kinetics of expression of genes that are dynamically regulated during development and differentiation. We have integrated therein the addition of fetal pancreatic CM, containing the best combination of soluble factors known to promote pancreatic differentiation (Brolen et al., 2005; Leon-Quinto et al., 2004; Skoudy et al., 2004).

First, we used a lentivirus to establish ES cell lines that constitutively express Pdx1. Because all the pancreatic epithelial cell types originate from Pdx1-expressing progenitors, this strategy might be used for the generation of both exocrine and endocrine differentiated cells (Gu et al., 2002). Nuclear expression of Pdx1 was detected in undifferentiated cells as well as in their differentiated derivatives up to the 30th day of differentiation (Fig. 1C, and data not shown). Although all ES cell lines were clonally derived, Pdx1 expression in undifferentiated ES cells was heterogenous, reflecting a common observation in ES cells due to vector integration into an ectopic site. After spontaneous differentiation of ES cells, Pdx1 increases the expression of a large number of acinar enzymes. This effect is likely mediated—at least in part—by the PTF1 complex, as these changes parallel an increase in p48/PTF1a (Fig. 3A), as occurs in vivo (Petrucco et al., 1990). However, the induction of acinar genes did not follow a single unique pattern: for example, carboxypeptidase A expression was not activated even when cells were cultured with fetal pancreatic CM. Several hypotheses can explain this result. First, the requirements for transcriptional activation may be slightly different at the promoters of the various exocrine-specific genes: the PTF1 complex is not sufficient to regulate the expres-

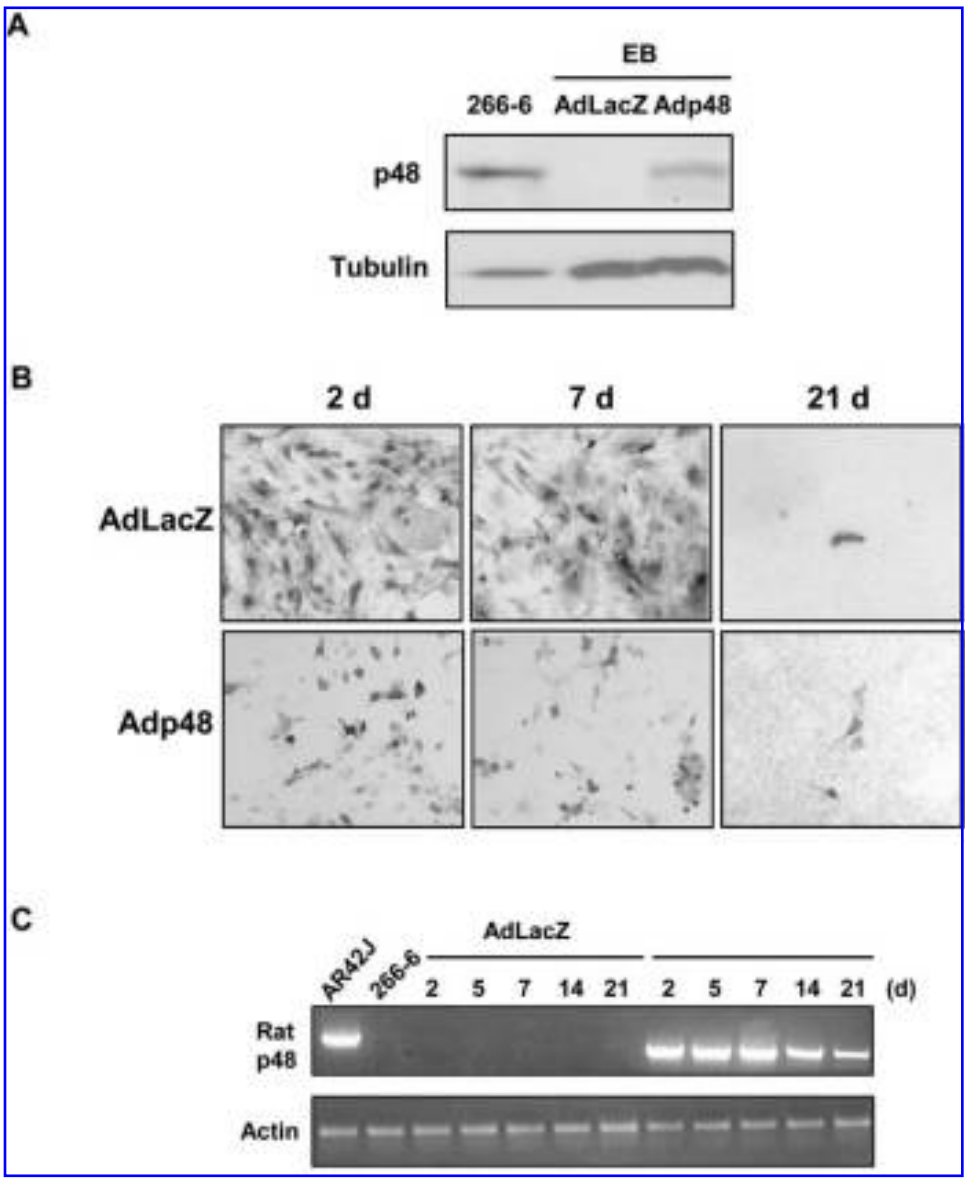

FIG. 5. Kinetics of transgene expression in differentiating ES4Pdx1 cells infected with rat p48- and LacZ-encoding adenoviruses. (**A**) Western blot analysis of p48 expression. Positive control, 266–6 cells. (**B**) Differentiated ES4Pdx1 cells infected with Adp48 or AdLacZ were immunostained with an anti-p48 antibody or assayed for β-galactosidase activity, respectively, at the indicated times after infection. Original magnification $\times$200. (**C**) RT-PCR analysis of total RNA from samples obtained as in **B** using primers specific for rat p48 and β-actin. Pancreatic 266–6 and AR42J cells were used as control of mouse and rat specificity, respectively.

sion of acinar genes, requiring the cooperation of HNF3β/HNF3γ or the complex composed by Pdx1, Meis2b, and Pbx1b (Adell et al., 2000; Cockell et al., 1995; Rose et al., 2001). A comparison of the promoter/enhancer sequences of acinar genes shows both conservation and divergence (unpublished observations). Second, it is possible that lack of carboxypeptidase A induction reflects the ability of Pdx1 to commit cells to an immature stage of differentiation. Third, carboxypeptidase A-expressing cells may be lost during culture. This is unlikely since positive cells are detected after infection with the Adp48 using similar cell culture conditions (Fig. 6, and not shown). However, we cannot completely rule out that these results reflect individual effects on each gene rather than the activation of a "differentiation programme."

There is cumulative evidence indicating that the expression of a single transcriptional activator is generally insufficient to activate a complete developmental process. Instead, a combinatorial

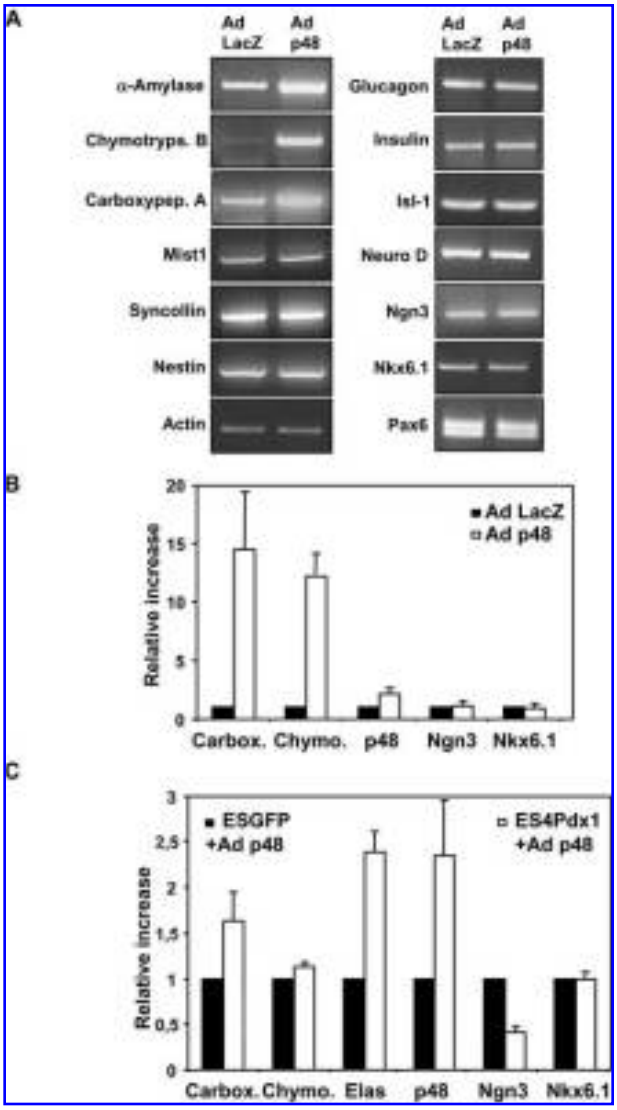

FIG. 6. Effect of p48 on gene expression of differentiating ES4Pdx1 cells. (**A**) ES4Pdx1 cells cultured as in Figure 3 were infected with Adp48 or AdLacZ and total RNA subjected to semiquantitative RT-PCR analysis for specific pancreatic markers. (**B**) Quantitative RT-PCR analysis for the expression of selected pancreatic markers using the RNA samples obtained in **A**. Histograms show the relative expression levels normalized to the loading control Hptr. (**C**) Quantitative RT-PCR was done as in **C** using the RNA of differentiated Adp48-infected ESGFP and ES4Pdx1 cells. Error bars indicate the standard deviations of two experiments performed in triplicate.

network acts to determine cell identity and function. The main novelty of our study relies on the simultaneous introduction of two cDNAs encoding transcription factors in differentiating ES cells using vectors with distinct transduction properties, in conjunction with stimulation with soluble factors. We chose to express p48 in Pdx1-transduced ES cells as the former is expressed in pancreatic progenitors and is required for acinar differentiation (Kawaguchi et al., 2002; Rose et al., 2001). p48 is expressed at E9.5–E10.5 (Kawaguchi et al., 2002; Krappe et al., 1998), after Pdx1 (Offield et al., 1996). Thus, ES4Pdx1 cells were infected with a p48-expressing adenovirus resulting in the selective expression of a large panel of acinar markers, but not endocrine markers (Fig. 6). The upregulation of some acinar genes (i.e., carboxypeptidase A) occurred only when p48 was overexpressed, possibly reflecting a dose-dependent effect. Interestingly, exogenous p48 upregulated endogenous p48 expression, suggesting that the modulation in the levels of expression of digestive enzymes results from a bona fide activation of the acinar differentiation program.

When p48 was transduced into control ESGFP, the induction of exocrine genes was very inefficient (Fig. 6C), demonstrating the cooperation of Pdx1 and p48. This effect likely relies on a regulatory loop involving both factors, as endogenous p48 expression was only activated in Pdx1-overexpressing cells.

Other important genes involved in acinar development were not induced (i.e., Mist1), suggesting that we have established immature exocrine cells. This tenet is further supported by the lack of regulation of syncollin mRNA, encoding a granule-associated protein required for exocytosis in exocrine cells (Fig. 6) (Wasle et al., 2005). We speculate that overexpression of additional key transcription factors may support a more differentiated phenotype.

Regarding the effects of Pdx1 on endocrine differentiation of ES cells, other groups have described either a modest effect or the expression of a set of genes involved in beta-cell development (Blyszczuk et al., 2003; Miyazaki et al., 2004). In our hands, ectopic Pdx1 tended to diminish the levels of several genes expressed in endocrine cells (Fig. 3). Of importance, the differentiation protocol that we have used is not based on the selection of nestin-expressing cells as used in other studies (Blyszczuk et al., 2003; Miyazaki et al., 2004). Indeed, regardless of the conditions assayed—use of fetal pancreatic CM (Fig. 3), expression of Pdx1 (Fig. 3), and coexpression of p48 (Fig. 6)—we failed to observe changes in nestin mRNA. Thus, we propose that our experimental protocol favors exocrine precursor generation. Alternatively, our data may reflect a dose-dependent effect of Pdx1: as shown in Figure 1A, ES4Pdx1 cells express lower Pdx1 levels than

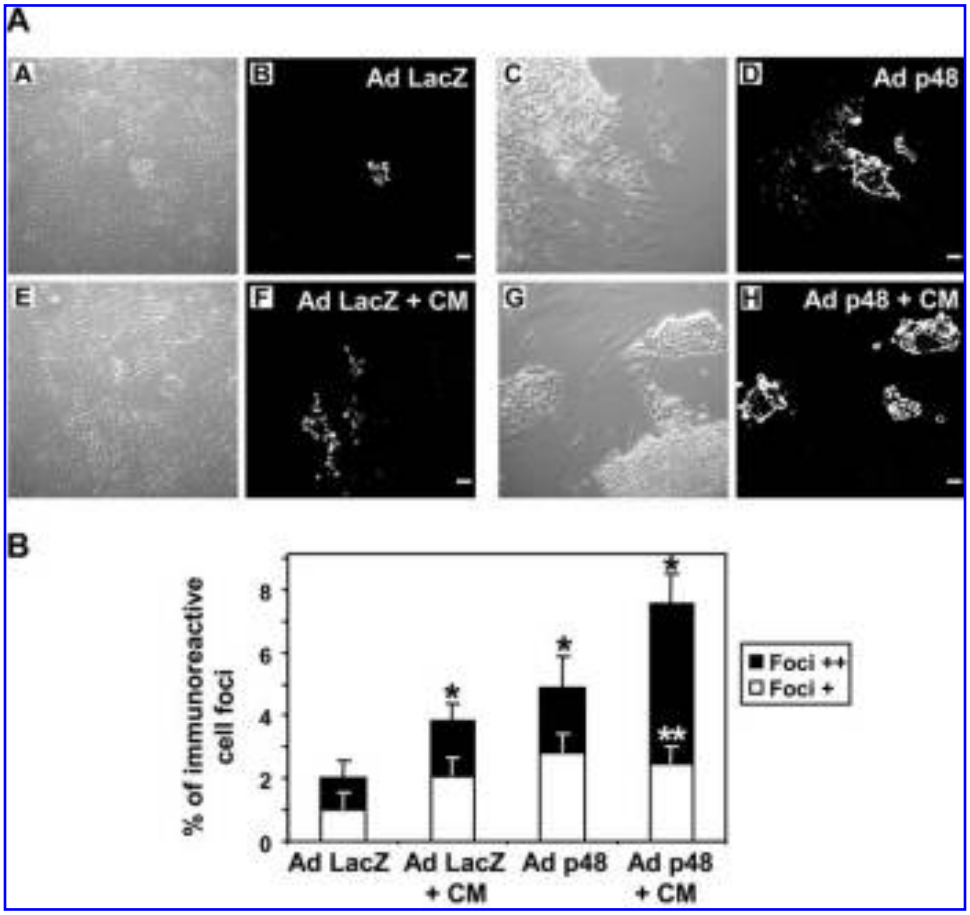

FIG. 7. Immunofluorescence analysis of differentiated ES4Pdx1 cells infected with rat p48 and LacZ expressing adenoviruses. (**A**) ES4Pdx1 cells incubated with (**F**, **H**) or without (**B**, **D**) CM were infected with AdLacZ (**B**, **F**) or Adp48 (**D**, **H**) and subjected to immunofluorescence analysis with an anti-amylase antibody. The corresponding phase-contrast microphotographs are shown in adjacent panels. Scale bars = 25 μm. (**B**) Histograms of data from one experiment representative of two performed indicating the percentage of immunoreactive cell foci, as well as their intensity, in the different cell culture conditions, as shown in **A**. + refers to the level of intensity of the immunostaining. Percentage of immunoreactive cells (foci + and foci ++) increase in comparison to control AdLacZ (*$p < 0.05$). Percentage of ++ foci increase in comparison to Adp48 (**$p < 0.05$). Both experiments yielded similar results.

266–6 cells. *In vivo*, Pdx1 levels are lower in acinar than in beta-cells, and targeted overexpression in exocrine pancreas leads to a severe exocrine dysmorphogenesis (Heller et al., 2001; Smart et al., 2005). The low Pdx1 levels achieved in our system may favor the exocrine commitment of pancreatic precursors. It will be important to discern among these possibilities in order to improve methods that selectively lead to the development of exocrine versus endocrine precursors. Finally, our data are very similar to those obtained in the *Xenopus* model. In this study, ectopic p48 allows the expression of exocrine markers in the Pdx1-expressing domains of the endoderm. In addition, coexpression of both factors in nonpancreatic domains at early stages leads only to exocrine pancreas formation, whereas the endocrine pancreas does not develop (Afelik et al., 2006).

In summary, we have developed methods to direct ES cells to acquire features of pancreatic acinar cells. The strategy has led to the discrimination of distinct stages of acinar differentiation that have not thus been reported. Our strategy efficiently allows to screen for the best nuclear regulator combinations to commit ES cells into the lineage of interest integrated with the use of soluble factors. Because adenoviral infection can be readily performed in mouse ES cells, the strategy is amenable to mid-throuput assays. Our work should lead to the *in vitro* generation of functional acinar cells suitable for functional replacement assays.

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