Murine Embryonic Stem Cell–Derived Pancreatic Acinar Cells Recapitulate Features of Early Pancreatic Differentiation

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Background & Aims: **Acinar cells constitute 90% of the pancreas epithelium, are polarized, and secrete digestive enzymes. These cells play a crucial role in pancreatitis and pancreatic cancer. However, there are limited models to study normal acinar cell differentiation in vitro. The aim of this work was to generate and characterize purified populations of pancreatic acinar cells from embryonic stem (ES) cells.** ***Methods:*** **Reporter ES cells (Ela-pur) were generated that stably expressed both β-galactosidase and puromycin resistance genes under the control of the elastase I promoter. Directed differentiation was achieved by incubation with conditioned media of cultured fetal pancreatic rudiments and adenoviral-mediated co-expression of p48/Ptf1a and Mist1, 2 basic helix-loop-helix transcription factors crucial for normal pancreatic acinar development and differentiation.** ***Results:*** **Selected cells expressed multiple markers of acinar cells, including digestive enzymes and proteins of the secretory pathway, indicating activation of a coordinated differentiation program. The genes coding for digestive enzymes were not regulated as a single module, thus recapitulating what occurs during in vivo pancreatic development. The generated cells displayed transient agonist-induced Ca^{2+} mobilization and showed a typical response to physiologic concentrations of secretagogues, including enzyme synthesis and secretion. Importantly, these effects did not imply the acquisition of a mixed acinar-ductal phenotype.** ***Conclusions:*** **These studies allow the generation of almost pure acinar-like cells from ES cells, providing a normal cell-based model for the study of the acinar differentiation program in vitro.**

Pancreatic acinar cells play a key role in digestion in vertebrates. The synthesis and secretion of digestive enzymes is finely regulated: many enzymes are synthesized as propeptides and stored in zymogen granules (ZG), the exocytosis of which is tightly controlled. Binding of secretagogues to the muscarinic 3 and cholecystokinin (CCK) receptors leads to Ca^{2+} release from the endoplasmic reticulum and triggers ZG secretion.[1]

Acinar cells also play an important role in exocrine pancreatic diseases, including acute and chronic pancreatitis and, possibly, ductal adenocarcinoma. Upon stress, acinar cells readily undergo a phenotypic switch, resulting in loss of differentiation and acquisition of duct-like features both in vivo and in vitro.[2–6] Together with their minimal proliferative potential, these effects hamper the study of acinar differentiation. Furthermore, there are very few acinar tumor cell lines and they lack differentiated features (ie, ZG). Because of their tumor origin, their study is also of questionable physiologic relevance. Therefore, the establishment of normal-cell–derived acinar cultures remains a high priority to better understand and manipulate acinar differentiation. In particular, it is important to better characterize and dissect substages of acinar differentiation during pancreatic development and regeneration to better interfere with their loss of differentiation properties. For instance, it generally is considered that genes coding for digestive enzymes are activated simultaneously and that their expression is under identical regulatory control mechanisms, yet conventional reverse-transcription polymerase chain reaction (RT-PCR) data support the notion that these genes are regulated sequentially.[7] Because nonquantitative RT-PCR (non–qRT-PCR) has technical limitations, the relative levels of expression of the genes coding for acinar enzymes at different developmental stages remains to be analyzed. This is important because in vivo tracing experiments evaluating the contribution of acinar cells to

Abbreviations used in this paper: Ad, adenovirus; Amyl, α-amylase; CPA, carboxypeptidase A; CCK, cholecystokinin; ChymoB, chymotrypsinogen B; CM, conditioned medium; EB, embryoid bodies; Ela1, elastase 1; ES, embryonic stem; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; ZG, zymogen granules.

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other pancreatic cell types currently are performed using digestive enzyme gene promoters.

Acinar cells originate from multipotent precursors located in the foregut giving rise to all pancreatic cell types. In mice, exocrine pancreas specification occurs at embryonic day 10 (E10). Cells with acinar features are distinguished at E14, ZGs accumulate from E16 to birth, and full maturation occurs postnatally.[8–10] However, little is known about the precise molecular events heralding acinar differentiation. To date, few transcription factors regulating exocrine differentiation have been identified: the basic helix-loop-helix proteins p48/Ptf1a and Mist1, and RBPL. Ptf1a messenger RNA (mRNA) is first detected around E9.5 in pancreatic primordia and, in the adult, becomes restricted to acinar cells. Ptf1a originally was described as part of the heterotrimeric transcription complex PTF1 involved in digestive enzyme gene expression.[11,12] Ptf1a also is essential for pancreas formation and for the development of all pancreatic cell lineages.[13–15] In its absence, foregut endoderm precursors assume an intestinal fate.[14] Ptf1a also promotes ectopic pancreas fates from endoderm tissue.[16–18] Mist1 is expressed in a wide array of secretory tissues and, in the adult pancreas, is detected only in acinar cells.[19,20] *Mist1* inactivation or inhibition of Mist1 function results in a severe impairment of acinar organization, including loss of gap junctions, structural alterations of secretory granules, and acinar-ductal metaplasia.[20,21] Therefore, Mist1 is necessary for proper cell polarization and maintenance of acinar identity. RBPL is essential for the high transcriptional activity of the PTF1 complex independently of Notch signaling.[22] Interestingly, RBPL is expressed in the developing pancreas tips at E14.5,[23] when exocrine precursors expand and differentiate, confirming a role in acinar differentiation during the secondary transition.[24]

Embryonic stem (ES) cells, derived from the inner-mass of pre-implantation embryos, can differentiate into cells of the 3 germ layers, making them an excellent tool for differentiation studies in vitro. Via embryoid body (EB) formation, endodermal precursors can be specified into endocrine and exocrine lineages in a process that partially recapitulates early pancreatic development.[25,26] Despite promising advances,[27] this process is very inefficient. Here, we aimed to generate ES cells that had activated a pancreatic acinar differentiation program to apply this knowledge to study exocrine pancreatic diseases. Our strategy relied on the ability of ES cells to respond to soluble factors involved in pancreatic embryogenesis in conjunction with expression of multiple exocrine transcription factors and genetic selection. This system allowed us to isolate normal cell populations displaying immature acinar phenotypes. These cells reproduce specific substages of acinar cell differentiation and should be valuable for studying the exocrine differentiation program.

Materials and Methods

ES Cell Culture and Transfection

CGR8 ES cells were maintained as published.[25] A reporter gene construct containing the -500/+8EI rat elastase I (Ela1) enhancer/promoter[28] was generated by subcloning the -500 fragment-driven puromycin resistance complementary DNA (cDNA) into pKS plasmid. An IresLacZ-mouse phosphoglycerate-kinase promoter-driven hygromycin resistance cassette (a gift from E. Maandag, The Netherlands Cancer Institute, Amsterdam, The Netherlands) was inserted downstream. Cells were electroporated (260V, 500 μF) using a Bio-Rad (Hercules, CA) gene pulser and selected using 200 μg/mL hygromycin to establish Ela-pur-IresLacZ clones (Ela-pur). Pancreatic acinar tumor cells (AR42J and 266-6) as well as NIH3T3 cells, were transfected as controls.

Adenoviral Generation and Gene Transduction

Adenoviral vectors were obtained as described.[29] Briefly, full-length cDNAs encoding rat Ptf1a[30] or mouse Mist1[31] were inserted into the pAd-shuttle-cytomegalovirus vector[32]; recombinant adenoviruses were produced by the Laboratory of Gene Therapy (Gene Vector Production Network, Nantes, France) using the Ad-Easy system.[32] Infections were performed as described in the Supplementary Materials and Methods section (see Supplementary material online at www.gastrojournal.org).

In Vitro Differentiation and Selection Procedure

To direct differentiation, Ela-pur ES clones were allowed to aggregate in bacterial Petri dishes (3.3×10^4 cells/mL) in medium supplemented with 3% fetal bovine serum without leukemia inhibitory factor. After 7 days, 30–50 EBs were plated in gelatin-coated, 6-well culture dishes for 7 additional days in 10% fetal bovine serum–supplemented medium (*differentiation step*). In some experiments, conditioned medium (CM) from E16.5 fetal pancreas cultures was added during this 14-day period (1:1 dilution with normal medium).[25] Medium was changed every 2 days. In addition, cells were infected 36 hours after EB plating with adenoviruses as described in the Supplementary Materials and Methods section. For ES–Ela-pur selection, differentiated cultures were maintained in differentiation medium plus puromycin (0.8 μg/mL) (Calbiochem, Darmstadt, Germany). After 2 weeks of selection, cells were re-infected with adenoviruses and cultured for an additional week (*selection step*, 35 days).

Molecular, Ultrastructural, and Functional Characterization of Selected Ela-Pur Cells

Cells were processed by immunocytochemistry, X-Gal staining, and electron microscopy as described

in the Supplementary Materials and Methods section. Expression analyses were performed by RT-PCR. Functional assays included bromodeoxyuridine incorporation, cytosolic Ca^{2+} measurements, and α-amylase (Amyl) secretion (see the Supplementary Materials and Methods section).

Statistics

Statistical differences were analyzed by the Student *t* test.

Results

Digestive Enzyme Gene Expression Is Not Activated as a Single Regulatory Module During Pancreatic Development

Ninety percent of acinar mRNA encodes a small number of enzymes, widely used as cytodifferentiation markers. However, little is known about the quantitative regulation of expression during mouse pancreatic development after initiation of acinar differentiation. We assessed the relative levels of selected mRNAs using quantitative RT-PCR and RNA from E12.5 pancreas until the postnatal period. We found that acinar gene expression does not follow a single pattern (Figure 1*A*). After an increase in levels of all digestive enzyme mRNAs at E14.5, distinct patterns were identified (Figure 1*A*). This diversity is better reflected by comparing changes in mRNA levels of chymotrypsinogen B (ChymoB), Amyl, and Ela1 with those of carboxypeptidase A1 (CPA). We chose CPA as a reference because it is the earliest enzyme transcript detected during development.[23] The CPA:ChymoB ratio remained essentially unchanged from E15.5 to adulthood (Figure 1*B*), indicating similar regulation. By contrast, the CPA:Amyl and CPA:Ela1 ratios (Figure 1*B*) decreased markedly over time, indicating that Ela1 and Amyl mRNAs undergo major up-regulation at later developmental stages. Moreover, the increase in Ela1 expression occurs mainly in the adult compared with postnatal day 2 (Figure 1). These regulatory patterns can be used to better characterize acinar differentiation in vitro.

Genetic Selection of ES Cells Engaging the Acinar Differentiation Program

Differentiating ES cells recapitulate many aspects of embryonic development in vivo; therefore, reporter ES clones were generated to study acinar differentiation. Undifferentiated ES cells were transfected with a construct conferring puromycin-resistance and β-galactosidase expression under the control of the rat Ela1 -500 enhancer[28]; the construct also contains a phosphoglycerate-kinase-Hygro unit for selection of transfectants (Figure 2*A*). Four hygromycin-resistant clones (Ela-pur) of undifferentiated ES cells screened by PCR to verify transgene integration (see the supplementary Materials and Methods section and supplementary Figure 1; see supplementary material online at www.gastrojournal.org) showed similar growth properties as parental cells and displayed similar expression profiles on differentiation (not shown). Because Ela1 already is expressed at E12 of development (Figure 1), this system will be useful to trace activation of acinar gene expression.

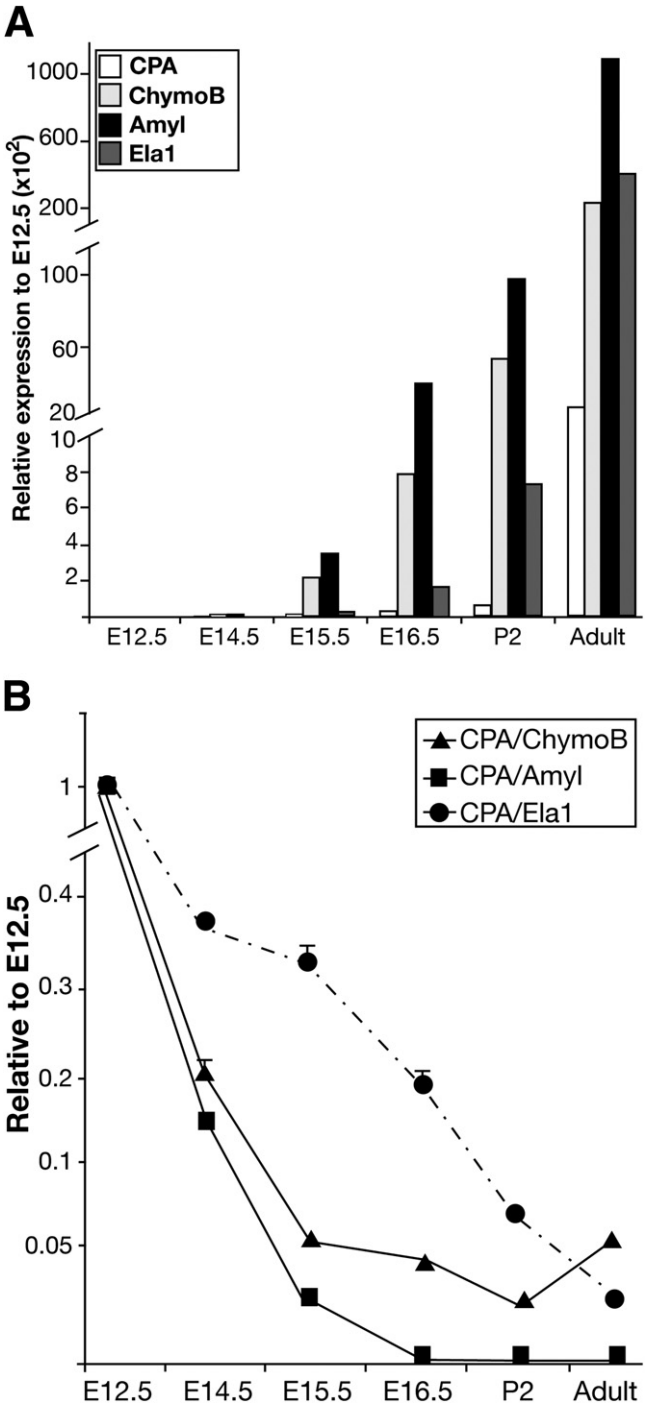

Figure 1. Expression of transcripts coding for digestive enzymes during pancreatic development and in adult pancreas assessed by qRT-PCR. (*A*) mRNAs from embryonic pancreatic rudiments and adult pancreas were quantified and transcript levels are expressed relative to those in the E12.5 pancreas. (*B*) Ratios of mRNAs levels of ChymoB, Amyl, and Ela1 relative to CPA mRNA levels (*A*) during pancreatic development.

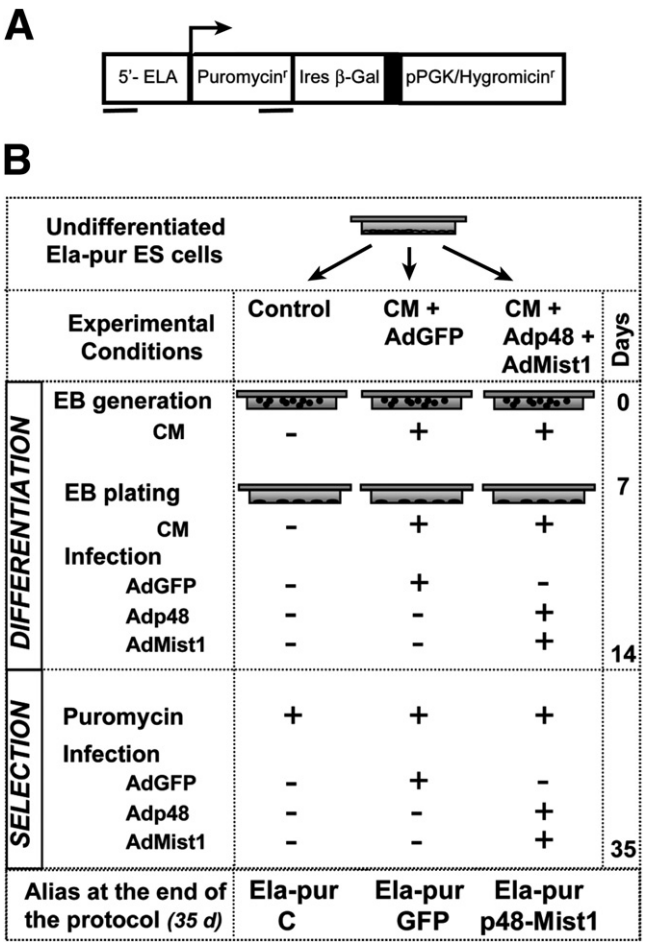

Figure 2. Generation of the reporter Ela-pur ES cell clones and the differentiation and selection protocol. (*A*) Structure of the Ela promoter-puro^r-IresLacZ- phosphoglycerate-kinase-Hygro^r construct. (*B*) Scheme of the protocol and experimental conditions used. Ela-pur cells were differentiated in suspension as EB for 7 days, plated in cell culture dishes, and grown for 7 days. During this period, adenoviral infections were performed in adhered EB and cultures were supplemented with CM from the culture of E16.5 fetal pancreatic rudiments, as indicated. For the sake of simplicity, this step was designated as *differentiation*. Cells having activated an acinar differentiation program were selected by incubation with puromycin. Two weeks later, cells were re-infected with the same adenoviruses and cultured for an additional week. This second step was designated as *selection*, although differentiation-related events could take place during this phase.

Differential Regulation of Digestive Enzyme Gene Expression by Ptf1a and Mist1 in Differentiating Ela-Pur ES Cell Clones

Soluble signals present in CM from E16.5 pancreas cultures, superimposed to adenoviral-mediated expression of Ptf1a, result in a synergistic induction of exocrine genes in differentiating ES cells.[29] We therefore investigated the effect of the combined transduction of Ptf1a and Mist1 on acinar gene expression because both transcription factors are co-expressed in pancreatic cells starting at E10.5. EBs from the Ela-pur clones were generated in the presence of CM and after 1 week were allowed to adhere for 7 days (differentiation step) (Figure 2*B*). In addition, EBs were infected with adenovirus expressing either GFP cDNA (AdGFP), or rat Ptf1a (Adp48) and mouse Mist1 (AdMist1),[29] resulting in 60%–70% of cells expressing transgenes (supplementary Figure 2*A*–*C*; see supplementary material online at www.gastrojournal.org). Control cells were generated by inducing spontaneous differentiation without additional experimental manipulation (Figure 2*B*). Importantly, qRT-PCR showed a differential regulation of acinar genes by Ptf1a and Mist1 at this stage: Ptf1a induced a marked increase in CPA but not in Ela1 mRNA whereas Mist1 did not have any effect on gene expression on its own. By contrast, the combination of both led to a significantly higher increase of CPA mRNA levels ($P = .003$ vs control) and, importantly, to a 2.5-fold increase in Ela1 mRNA levels (Figure 3). Therefore, Ptf1a and Mist1 activated a gene expression pattern similar to that of early exocrine cells (Figure 1). Because Ela1 mRNA was enhanced exclusively in this condition, it was chosen for further studies.

To select for cells having activated an acinar differentiation program, puromycin was added and cells were re-infected with both Adp48 and AdMist1 (Figure 2*B*). Transient sequential gene expression obtained with adenoviruses, rather than sustained expression, was used to avoid the strong antiproliferative effect of Ptf1a[30]; multiplicity of infection was optimized to favor cell survival, leading to 40% of the cells infected at this stage (supplementary Figure 2*E*–*G*; see supplementary material online at www.gastrojournal.org). Cells were analyzed after 3 weeks of selection, when isolated colonies had expanded (n = 8) (selection step, Figure 2*B*). Undifferentiated Ela-pur ES cells did not survive puromycin selection (n = 3). After inducing differentiation with CM and Ptf1a and Mist1 adenoviruses (referred to as *Ela-pur$^{p48\text{-}Mist1}$* after selection), greater than 95% cells were strongly β galactosidase positive (n = 6) (Figure 4*A*). In conclusion, cell-trapping results in an efficient selection of elastase-expressing cells.

Cells Having Activated the Acinar Gene Expression Program Display a Reduced Proliferative Ability

To characterize the features of the acinar differentiation program in genetically selected cells, qRT-PCR was used (Figure 4*B* and *C*). The highest digestive enzyme mRNA levels were achieved in Ela-pur$^{p48\text{-}Mist1}$. Interestingly, only CPA and ChymoB increased up to 300-fold in comparison with spontaneously differentiated and selected cells (Ela-pur^C) (Figure 4*B*). The ratio of CPA/ChymoB mRNA levels was 0.43, a value similar to that in early (E12.5–E14.5) pancreatic development (Figure 1*B*). Importantly, these effects were associated with an increase in endogenous Ptf1a and RBPL mRNAs, but not of endogenous Mist1 (Figure 4*C*). As expected, high

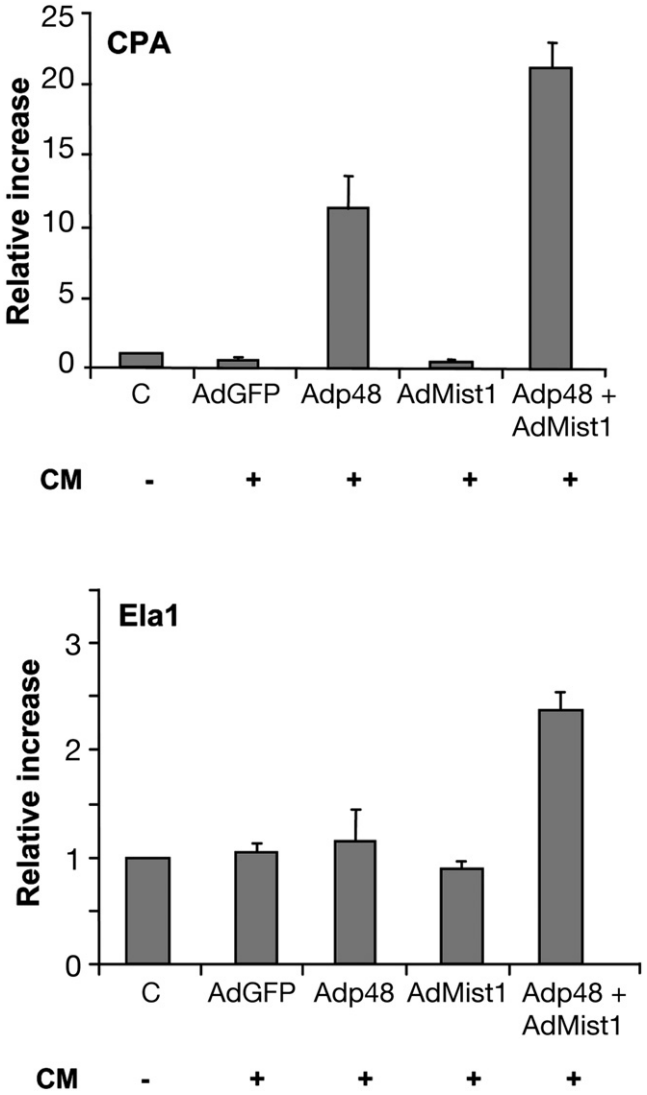

Figure 3. Expression of digestive enzyme transcripts after the differentiation step by qRT-PCR. mRNA transcripts were analyzed just after the first step of differentiation as described in Figure 2*B*. After EB adhesion, cells were infected—or not—with GFP cDNA (AdGFP), Adp48, AdMist1, or with a combination of both Adp48 and AdMist1. *Error bars* indicate the standard deviations of 2 independent experiments.

levels of ectopic Mist1 mRNA were detected (supplementary Figure 3; see supplementary material online at www.gastrojournal.org). Pdx1, a transcription factor involved in both exocrine and endocrine differentiation, was expressed—but not significantly modulated—in these conditions (Figure 4*C*), possibly reflecting the low Pdx1 levels characteristic of acinar cells.[33,34] In addition, expression of selected genes whose products are involved in acinar secretion was analyzed (Figure 4*C*). Ela-pur$^{p48\text{-}Mist1}$ cells expressed mRNAs coding for cholecystokinin A receptor (CCKAR) and muscarinic 3 receptors as well as IP3R3, an important signaling mediator. Transcripts coding for connexin 32, a gap junctional protein required for exocrine secretion,[35] also were detected. By contrast, endocrine marker levels were very low (Ngn3, Nkx6.1) (supplementary Figure 4; see supplementary material online at www.gastrojournal.org) or undetectable (insulin). Cytokeratin 19 (CK19) and cystic fibrosis transmembrane conductance regulator (CFTR) (ductal) mRNA levels were not modulated (Figure 4*C*).

To assess whether physiologic cytodifferentiation was engaged, expression of selected corresponding proteins was studied using immunofluorescence. Ninety-eight percent of Ela-pur$^{p48\text{-}Mist1}$ cells expressed E-cadherin and β-catenin at cell–cell contact sites (results from 3 independent experiments), showing their epithelial nature (Figure 5*A* and *B*). Cytoplasmatic Amyl and CPA were detected in most cells (67% ± 3.8% and 80% ± 1.54%, respectively) (Figure 5*C* and *D*), as in AR42J cells (supplementary Figure 5*A* and *B*; see supplementary material online at www.gastrojournal.org). In agreement with the RT-PCR results, nuclear Pdx1 (Figure 5*E*) and RBPL (Figure 5*F*) were expressed. These proteins were undetectable in undifferentiated Ela-pur cells (Figure 5*C*' and *F*'). Regarding the secretory signaling pathway and machinery, CCKAR (Figure 5*I*), VAMP8—a SNARE protein associated with ZG whose function is crucial for secretion[36] (Figure 5*G*), and syntaxin 4—a membrane protein of pancreatic acinar cells[36] (Figure 5*H*), displayed membrane (Figure 5*H* and *I*) or cytoplasmic patterns (Figure 5*G*). By contrast, Dolichos biflorus agglutinin, which selectively recognizes ductal cells,[37] was restricted to very few cells (1.8% ± 1.82%) that were β-galactosidase negative (Figure 5*J*). Moreover, bromodeoxyuridine uptake assays showed that Ela-pur$^{p48\text{-}Mist1}$ cells showed reduced proliferative ability as compared with other experimental conditions (Figure 4*D*). In addition, cyclin D1 mRNA levels were reduced in these cells (Figure 4*E*). Collectively, these data show the coordinated activation of an acinar differentiation program in Ela-pur$^{p48\text{-}Mist1}$ cells.

Ela-pur$^{p48\text{-}Mist1}$ Cells Contain ZG and Respond to Acinar Secretagogues

A hallmark of acinar cells is enzyme storage in electron-dense ZG (Figure 6*A*). Ela-pur$^{p48\text{-}Mist1}$ cells displayed 250- to 600-nm ZG-like vesicles (Figure 6*B*), which were very scarce in spontaneously differentiated cells (Figure 6*C*), but present in selected CM-treated cells transduced with GFP (Ela-pur^{GFP}), suggesting that CM favors ZG formation and contributes to improved secretion to secretagogues (see below). Gold immunoelectron microscopy showed that these vesicles contain Amyl (Figure 6*E* and supplementary Figure 6; see supplementary material online at www.gastrojournal.org).

To further investigate their functional properties, cells were loaded with the calcium-sensitive dye fura-2 and the intracellular Ca^{2+} response to 5 μmol/L carbachol was monitored. Figure 7*A* summarizes results from 4 experiments. Ela-pur$^{p48\text{-}Mist1}$ cells (Figure 7*A*, *right*) displayed a rapid and transient increase in Ca^{2+} concentration, similar to that described for primary isolated mouse acinar

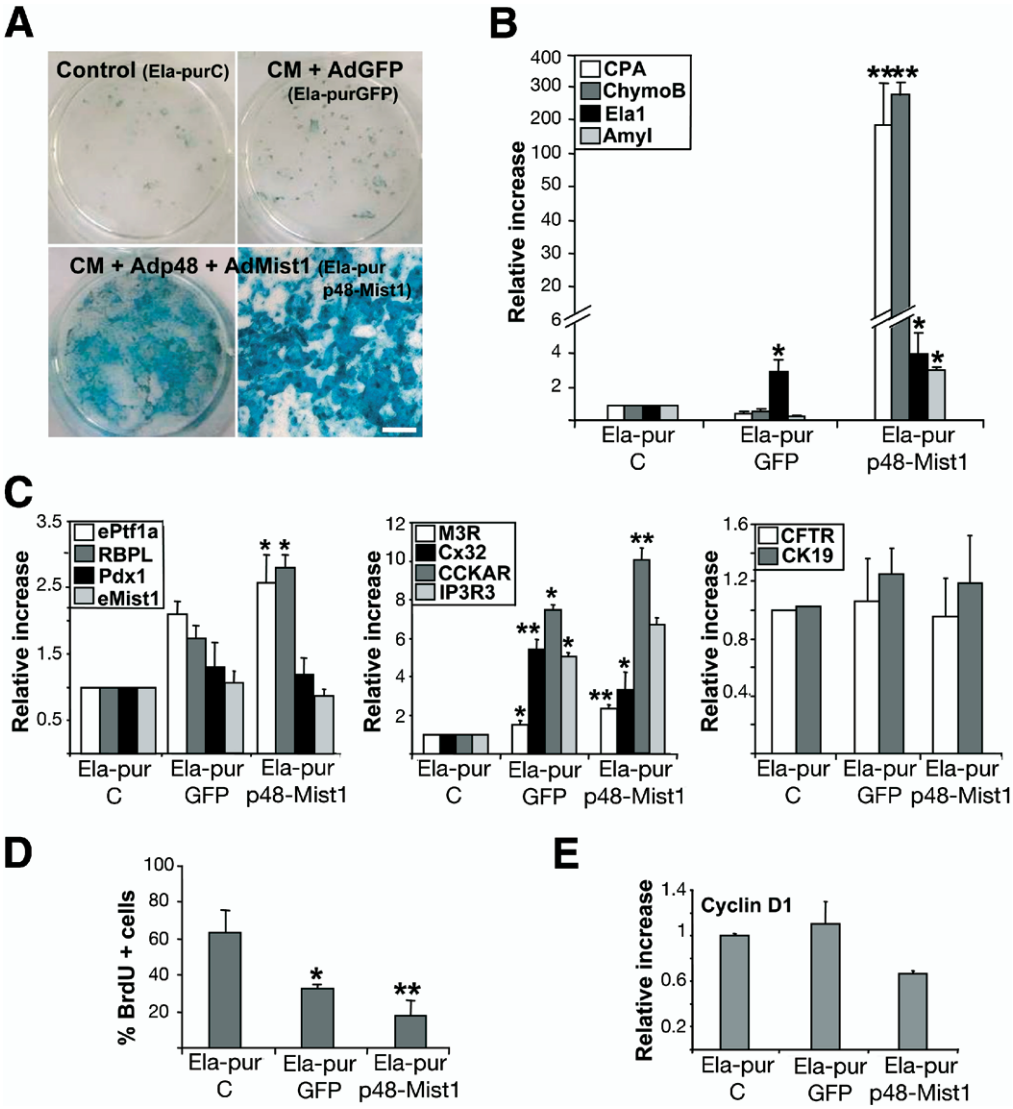

Figure 4. Selection of elastase-producing cells. (*A*) Analysis of the reporter β-galactosidase activity. X-Gal staining was performed after selection of differentiated cells. For each condition, representative staining of the selected colonies is shown. *Scale bar*, 100 μm. (*B*) Digestive enzyme and (*C*) exocrine gene expression by q-PCR. *Histograms* show the relative expression levels normalized to the loading control Hprt. *Error bars* indicate the standard deviations of 4 experiments in *B* and of 2–3 experiments in *C*. $^{*}P < .05$ and $^{**}P < .01$ compared with Ela-pur^{C} cells. (*D*) Bromodeoxyuridine (BrdU) incorporation in the differentiation conditions analyzed by fluorescence-activated cell sorter (n = 3). $^{*}P < .05$ and $^{**}P < .01$ compared with Ela-pur^{C} cells. (*E*) Cyclin D1 expression by q-RT-PCR analyzed as in *C*.

cells.[38] In contrast, Ela-pur^{C} cells (*left*) showed a smaller increase in Ca^{2+} concentration lacking the typical transientness of the normal response. Peak increases were statistically different for the 2 conditions ($P < .05$), whereas the plateau values at the end of the carbachol pulse were not different ($P > .05$). The percentage of carbachol-responsive cells was higher in Ela-pur$^{p48\text{-}Mist1}$ (35%; 97 of 280) than in Ela-pur^{C} (14%; 33 of 328). Ela-pur^{GFP} displayed an intermediate response (not shown).

In acinar cells, intracellular free Ca^{2+} is considered the primary trigger for enzyme secretion. Therefore, we analyzed the ability of cells to secrete Amyl in response to secretagogues in vitro (Figure 7*B*). Carbachol and CCK induced a significant increase in total Amyl activity (Figure 7*B*, *left*); this effect was more pronounced in Ela-pur$^{p48\text{-}Mist1}$ cells ($P < .05$ compared with Ela-pur^{C} cells), suggesting de novo synthesis of digestive enzymes mediated by the secretagogues, a known effect of these molecules. Both secretagogues also induced a significant increase in the extracellular Amyl activity (Figure 7*B*, *middle*), being more pronounced in Ela-pur$^{p48\text{-}Mist1}$ cells ($P < .05$). Altogether, these findings support the notion that both increased synthesis and secretion occur in response to secretagogues.

Discussion

Previous attempts to generate acinar cells in vitro, based on the improvement of primary culture conditions, have been unsuccessful. In all cases, acinar-ductal metaplasia/transdifferentiation occurs in association with a rapid loss of functional properties.[39–42] Here, we describe the development of immature acinar cells using the ES–EB model, which recapitulates many cues and signals required for the exocrine development in vivo. Our strategy takes advantage of the genetic selection of acinar lineage committed cells using an acinar-specific elastase promoter. The high efficiency of our strategy was confirmed by X-Gal

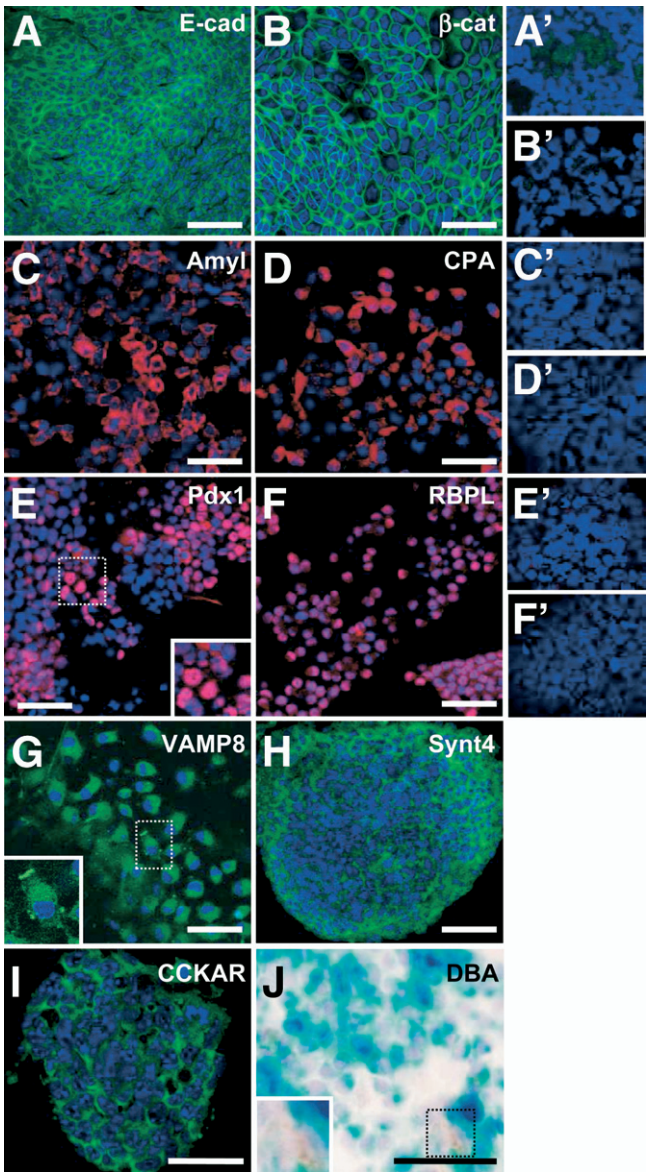

Figure 5. Immunofluorescence analysis of Ela-pur$^{p48\text{-}Mist1}$ cells. Confocal images of cells immunostained for (*A*) E-cadherin, (*B*) β-catenin, (*C*) Amyl, (*D*) CPA, (*E*) Pdx1, (*F*) RBPL, (*G*) VAMP8, (*H*) syntaxin 4, and (*I*) CCKAR. As negative controls, we show the staining without primary antibodies and with anti-mouse (*A'*) and anti-rabbit (*B'*) secondary antibodies on Ela-pur$^{p48\text{-}Mist1}$ cells, whereas *C'*, *D'*, *E'*, and *F'*, show the staining with the corresponding primaries antibodies on undifferentiated Ela-pur ES cells. Nuclei were labeled with ToPro-3 iodide (blue). (*J*) The *box* shows a brown cell stained for Dolichos biflorus agglutinin in double staining with X-Gal. *Scale bars*: *A–I*, 50 μm; *J*, 100 μm. Supplementary Figure 5 (see supplementary material online at www.gastrojournal.org) shows immunostaining of AR42J acinar cells with the same antibodies, for comparison.

staining, showing that nearly all cells expressed the reporter construct. Contamination by other cell types (ie, endocrine or ductal) was minimal (data not shown).

The ability to generate essentially pure acinar-like cells provides a powerful model to study acinar differentiation. For instance, this system has major potential to directly assess the functional role of candidate genes involved in exocrine development or function: cells selected on spontaneous differentiation indeed express Ptf1a and Mist1, although at levels that are much lower than found in cells transduced with Ptf1a and Mist1, providing evidence that regulation of transcription factor levels is a key step during acinar differentiation. In this regard, we found that endogenous Ptf1a also was up-regulated by co-expression of Ptf1a and Mist-1, suggesting the existence of an autoregulatory loop favoring exocrine differentiation. Indeed, such autoregulatory circuits have been described recently during exocrine pancreatic development. Thus, RBPL is able to autoregulate its expression as acinar differentiation progresses, providing a possible mechanism to ensure the maintenance of the acinar phenotype.[24] Interestingly, such positive regulation was not observed for Mist1 because endogenous mRNA levels were not modulated (Figure 4*C*), probably owing to the high levels achieved after gene transduction, which were found to be quite similar to those observed at E16.5 and in adult pancreas (Supplementary Figure 7; see Supplementary material online at www.gastrojournal.org). Furthermore, we showed that the combination of Ptf1a and Mist1 activates acinar cell gene expression in short-term experiments. In this sense, it has been described that *Mist1* knock-out mice show a reduced amount of Amyl during early stages of pancreatic development.[19]

The differentiated cells produced using this strategy showed a significant up-regulation of acinar gene expression as shown by qRT-PCR and immunocytochemistry. In particular, several digestive enzymes were up-regulated, reflecting not only an increase in the Ptf1a/p48-mediated PTF1 activity, but also the true activation of the acinar differentiation program. This notion is supported by the accompanying increase in RBPL, a key component of the PTF1 complex conferring its high activation potential. Importantly, the genes coding for digestive enzymes are not regulated as a single module, but instead display distinct regulatory patterns at different stages of acinar cell differentiation as suggested by earlier experiments.[7] In this sense, it was reported very recently that the ability of RBPL to regulate the expression of the zymogen genes is not universal.[24] Interestingly, it was proposed that a cell population expressing high levels of CPA corresponds to the main multipotent progenitor population during pancreatic development.[23] These cells, lacking expression of Amyl, are incompletely characterized. On the basis of our results showing a strong up-regulation of CPA and ChymoB expression, but not of Elas1 and Amyl, we suggest that ChymoB also could be used to trace early exocrine precursors.

In addition to generating a highly enriched population of cells expressing digestive enzymes and proteins involved in the secretory pathway, nearly 40% of the cells display functional properties of native acinar cells. It is intriguing that this figure is similar to the proportion of cells that could be transduced efficiently by adenoviral

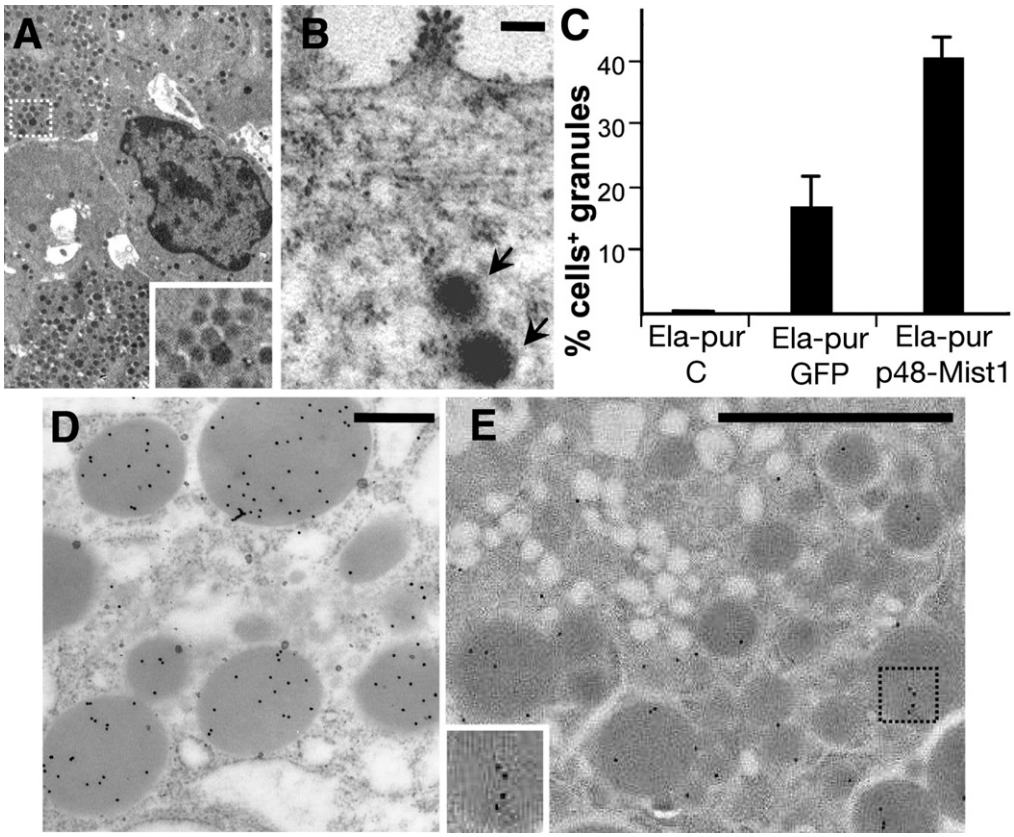

Figure 6. Immunoelectron microscopy analysis of Ela-pur$^{p48\text{-}Mist1}$ cells. Electron micrographs illustrating electron-dense vesicles in (*A*) murine pancreas (*inset*) and (*B*) Ela-pur$^{p48\text{-}Mist1}$ cells (*arrows*). (*C*) *Histograms* of data from 2 experiments showing the percentage of cells displaying these vesicles in the indicated culture conditions. Immunogold labeling with anti-Amyl antibody in (*D*) murine pancreas and (*E*) Elas-puro$^{p48\text{-}Mist1}$. *Scale bars*: *B*, 1 μm; *D*, 0.6 μm; *E*, 1.7 μm.

transgene delivery during selection. Because Ela-pur^{C} cells that differentiated spontaneously showed an aberrant pattern of Ca^{2+} response, similar to that described in acinar cells of Mist-1–deficient mice,[38] it is tempting to speculate that efficient expression of Mist1 renders the Ca^{2+} signaling machinery more mature in elastase-pro-

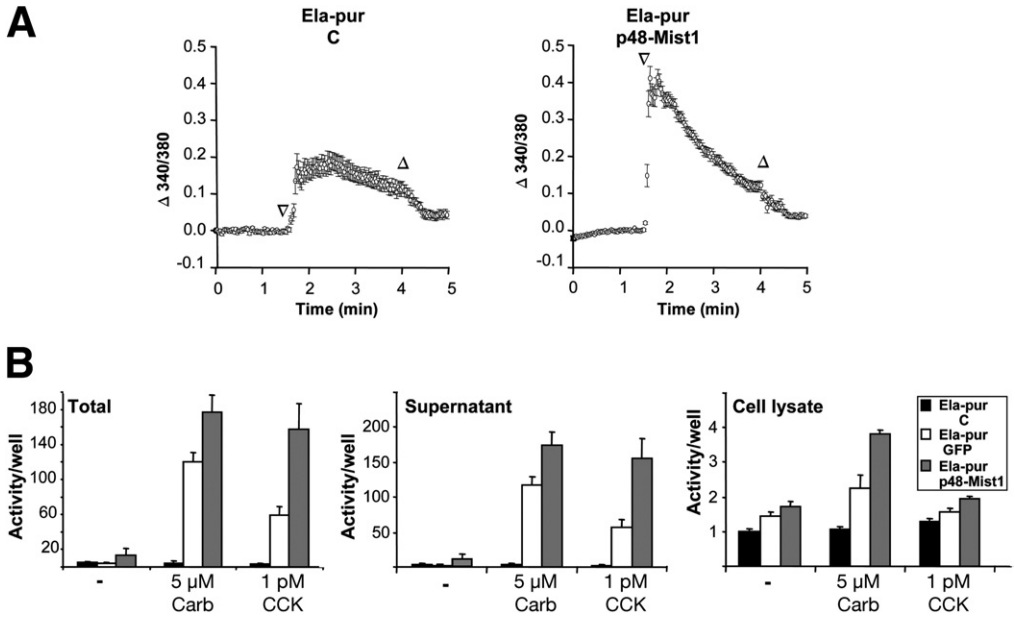

Figure 7. Carbachol-evoked Ca^{2+} signaling and exocytosis in Ela-pur selected cells. (*A*) Cytosolic Ca^{2+} signals in Fura-2–loaded cells stimulated with 5 μmol/L carbachol for 2.5 minutes. At the indicated times, the secretagogue was superfused and removed. Ela-pur^{C}, n = 33; Ela-pur$^{p48\text{-}Mist1}$, n = 97. (*B*) Selected cells were stimulated for 30 minutes with the indicated concentrations of carbachol and CCK. Amyl activity was measured in both the supernatant and cell lysates. Total activity corresponds to the sum of secreted and intracellular Amyl activities. *Error bars* indicate the standard deviation of 2 experiments performed in triplicate. ■, Ela-pur^{C}; □, Ela-pur^{GFP}; ▨, Ela-pur$^{p48\text{-}Mist1}$.

ducing cells, as has been proposed in vivo. Also, the percentage of cells showing ZG-like vesicles was higher in Ela-pur$^{p48\text{-}Mist1}$ cells than in any other experimental condition, in agreement with the fact that the number of ZGs is reduced in Mist1 knock-out mice.[19] Overall, we show that the directed-differentiated cellular response to Ptf1a and Mist1 is similar to early stages of pancreatic exocrine development, supporting the validity of our approach to develop functional genomic assays.

A major contribution of this work is that the cell population generated largely consists of cells lacking a mixed acinar-ductal phenotype, in contrast to reports based on the culture of purified pancreatic acinar cells in which a dedifferentiation process takes place. Because we have taken advantage of the fact that ES cells recapitulate embryonic developmental processes, we may have been able to avoid the signals that are triggered on mature acinar cell isolation and in vitro culture. The signaling pathways proposed to be activated under these conditions are mediated by Notch and epidermal growth factor transduction, which lead to the transdifferentiation of acinar cells to a ductal lineage.[2,3,40] We have not detected clear changes in the activation of the Notch signaling pathway in the culture conditions tested here (not shown), and the expression of Pdx1, which is induced during the acinar-ductal switch, also was not regulated.[40] Because acinar cells express much higher levels of digestive enzymes in the adult than during development, it is possible that only when committed cells terminally achieve a mature differentiation program are the protective mechanisms to bypass the lytic potential of their products activated. This fully differentiated state may include a higher potential of cell plasticity in vitro. An alternate explanation is that in vivo metaplasia occurs because of expansion of duct-like cells without dedifferentiation/transdifferentiation of acinar cells.[37]

The importance of the molecular characterization of acinar cell populations undergoing embryonic maturation has been emphasized by several recent experimental models showing that pancreatic ductal neoplasia can be induced by activation of oncogenes (ie, K-ras) in all cells of the pancreas[43,44] as well as in acinar cells.[6,45,46] Immature acinar cells are more susceptible to the transforming effects of K-ras activation than mature acinar cells in vivo.[45] Providing new insights into the identity of the immature acinar cells targeted during cancer development[45] is an important issue to understand the nature of pancreatic cancer precursors. Consequently, the identification of new markers distinguishing acinar cell populations during development and in disease conditions remains an important aim. Global transcriptome analyses of ES-derived exocrine cells should be informative and currently are underway in our laboratories.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.06.049.

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F.D. and M.M. contributed equally to this work.

Supplementary Material and Methods

Tissue Isolation

Murine embryonic pancreas isolation was performed under a stereomicroscope and tissues were transferred to RNAlater (Qiagen, Valencia, CA) for RNA extraction. All animal experiments were performed in accordance with the guidelines of the Animal Research Ethical Committee of our Institution.

Genomic PCR for Screening ES Stable Clones

PCR amplification on genomic DNA was performed for the detection of the elastase-puro^r transgene using EcoTaq (Ecogen, Barcelona, Spain) and the following primers: 5′-TTAACTGAGTGCCGGCCTT-3′ and 5′-AACTGCAGTGTGGTATGGCTGATTATGA-3′ that anneal to the 5′ region of the elastase I enhancer and the 3′ region of the puro^r cDNA, respectively.

Adenoviral Gene Transduction

Cells were washed 3 times with phosphate-buffered saline (PBS) and infected for 12 hours with recombinant adenoviruses at a multiplicity of infection of 1:25 in serum-free medium. Virus was eliminated by washing and cells were cultured further in medium supplemented with fetal bovine serum or with CM. Under these conditions, the viability of the cells was not compromised as scored by trypan blue dye exclusion in replicated wells.

qRT-PCR

RNA was prepared using the GenElute mammalian total RNA kit (Sigma, St. Louis, MO) and treated with DNase I (Ambion, Austin, TX). Real-time RT-PCR was performed in triplicate on an ABI Prism 7900HT Sequence Detector with the following TaqMan reagent kits (Applied Biosystems, Foster City, CA): Mm00481616_m1 for CPA, Mm02342486_mH for Amyl, Mm00481616_m1 for ChymoB, Mm00712898_m1 for Ela1, Mm00479622_m1 for Ptf1a, Mm00437606_s1 for ngn3, Mm00454962_m1 for nkx6.1, Mm01259683_g1 for insulin I, and Mm00446968_m1 for Hprt. Expression for other markers was performed with standard techniques and using the TaqMan RT reagents for retrotranscription, the quantitative SYBR Green PCR kit (Applied Biosystems), and the following primers: CK19 (S: CCTCCCGAGATTACAACCACT and AS: GGCGAGCATTGTCAATCTGT), CFTR (S: ACAAAACCTGGATCCCAATG and S: GAGCTGTCCAGGAAACTGCT), Pdx1 (S: AAATCCACCAAAGCTCACGC and AS: CGGTCAAGTTCAACATCACTGC), Cx32 (S: CCCACCGAGAAAACCGTCTT and AS: AGGCCCGGATGATGAGGTA), CCKAR (S: CTGTCCCTGGCTGTCAGTGA and AS: ACACGGCACTTCCGAAGATG), M3R (S: GAAGCGGAAGCAGAAAACTTTG and AS: TCCTCCTAGATGACCGTTTCGT), IP3R3 (S: CCTCCCCCAAGGCAAACT and AS: GACCTAGCATCCTCTAAGGCACTAA), RBPL (S: ACTCCGGTGCCTCTCATCAG and AS: CTACGCACACCAAGGAACGA), endogenous and ectopic Mist1 (S: GAACACCCATGCAGGACACAG and AS: CCTGGAAGGCATTGTTGAG), endogenous Mist1 (Mist1e) (S: TGAACTCCTGATCCTCCTC and AS: AGTTGAAGGCCCAGATCAC), cyclin D1 (S: CCCTCCGTATCTTACTTCAA and AS: GGAATGGTCTCCTTCATCTT) and Hprt (S: GGCCAGACTTTGTTGGATTTG and AS: TGCGCTCATCTTAGGCTTTGT). The data were processed using SDS 2.1 software and results were normalized to Hprt mRNA levels.

Immunocytochemistry

For the detection of adenoviral-mediated transgene expression, immunostaining was performed for 1 hour in PBS containing 1% bovine serum albumin with primary rabbit antibodies raised against Ptf1a[1] and Mist1. Cells were incubated with the Envision secondary reagent (Dako, Glostrup, Denmark) and the reactions were developed using 3,3'-diaminobenzidine tetrahydrochloride as a chromogen. This substrate also was used to detect the duct-specific lectin Dolichos biflorus agglutinin coupled to peroxidase (EY Laboratories, San Mateo, CA). Immunofluorescence staining was conducted using the Tyramide Signal Amplification method (TSA Fluorescence Systems; Perkin Elmer, Waltham, MA) and rabbit antibodies against Pdx1,[25,29] RBPL[2] (a kind gift from R. Wagener, University of Cologne, Germany), Amyl (Sigma), and CPA (Biogenesis, Sandown, NH). Alternatively, staining with mouse antibodies against E-cadherin (Transduction Laboratories, Lexington, KY), β-catenin (Transduction Laboratories), and rabbit antibodies against syntaxin 4 (Synaptic Systems, Göttingen, Germany), VAMP8 (Abcam, Cambridge, UK), and CCKAR (Euro-diagnostica, BN Arnhem, The Netherlands) was revealed using anti-IgG coupled to fluorescein (Dako). Nuclear labeling was performed with ToPro-3 iodide (Molecular Probes, Leiden, The Netherlands). Immunofluorescence staining and GFP expression were visualized with a Leica (Houston, TX) TCS-SP2 confocal microscope. In situ β-galactosidase activity was detected using standard procedures.

Bromodeoxyuridine Incorporation

Cells were incubated with 1 μmol/L bromodeoxyuridine (Sigma) for 16 hours, dissociated into single-cell suspensions, and resuspended in 300 μL of fixation and permeabilization solution (Cytofix/Cytoperm kit; BD Biosciences, San Diego, CA) for 20 minutes at 4°C. After washing with Perm/Wash buffer, the DNA was denatured with 2 mol/L HCl for 20 minutes. Immunofluorescence was performed following a standard protocol and using a mouse monoclonal antibody against bromodeoxyuridine (BD Biosciences) and an anti-mouse IgG coupled to fluorescein (Dako). Nuclear

staining was performed by incubating the cells with 5 μg/mL propidium iodine and 150 μg/mL RNase overnight at 4°C. Staining was analyzed by flow cytometry (Becton Dickinson).

Electron Microscopy

Cells were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Epon 812 resin as described.[39] Semithin sections of cell layers were examined after staining with toluidine blue. Thin sections were stained with uranyl acetate and lead citrate and examined using a Philips (Eindhoven, The Netherlands) 301 electron microscope. For immunogold labeling the anti-Amyl antibody, followed by a goat anti-rabbit antibody conjugated with 15-nm gold particles (Aurion, Wageningen, The Netherlands), were used. Samples were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS and embedded in Unicryl (Eppelheim, Germany).

Cytosolic Ca^{2+} Measurements

Intracellular Ca^{2+} concentration measurements were performed as described previously.[3] Cells were incubated in a bathing solution (140 mmol/L NaCl, 2.5 mmol/L KCl, 1.2 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$, 5 mmol/L glucose, and 10 mmol/L HEPES, pH 7.25 and 305 mOsm) containing 5 μmol/L Fura-2 AM for 45 minutes at room temperature and washed thoroughly with fura-2–free solution before initiating the experiment. Video microscopic measurements of $[Ca^{2+}]$ were obtained using an Olympus (Center Valley, PA) IX70 inverted microscope with a 40× oil-immersion objective, a Polychrome IV monochromator (Till Photonics), a digital charge-coupled device camera, and the AquaCosmos software program (both from Hamamatsu Photonics). Fluorescence ratio (340 nm/380 nm) images were computed every 2 seconds.

Amylase Secretion Assay

Cells were washed with PBS and fresh cell culture medium without fetal bovine serum was added, supplemented or not with cholecystokinin octapeptide (Sigma) or with carbachol at the indicated concentrations for 30 minutes at 37°C. Culture supernatants then were collected and cells were lysed in Krebs–Ringer buffer containing 0.2% bovine serum albumin. Amyl activity was determined using the Infinity Amylase Liquid Stable Reagent (Termo Electron, Woburn, MA). To normalize the amount of Amyl secretion, the total protein content was measured by the Bradford method. Amyl released into the supernatant and Amyl content of the cell pellets was determined. Each experiment was performed in triplicate. Positive control experiments were performed using isolated mouse pancreatic acinar cells (not shown).

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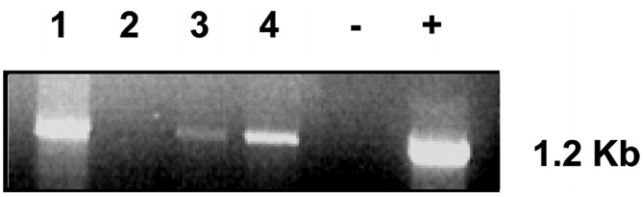
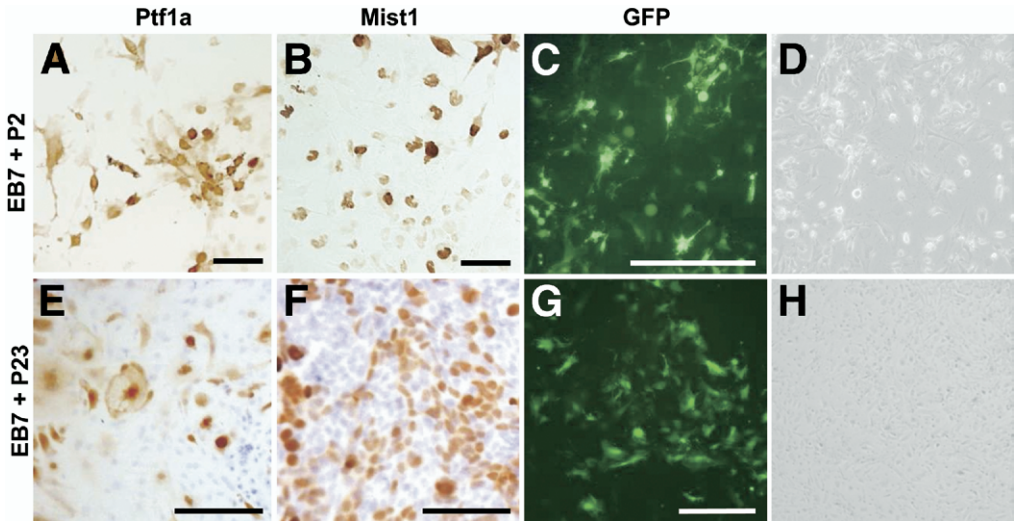
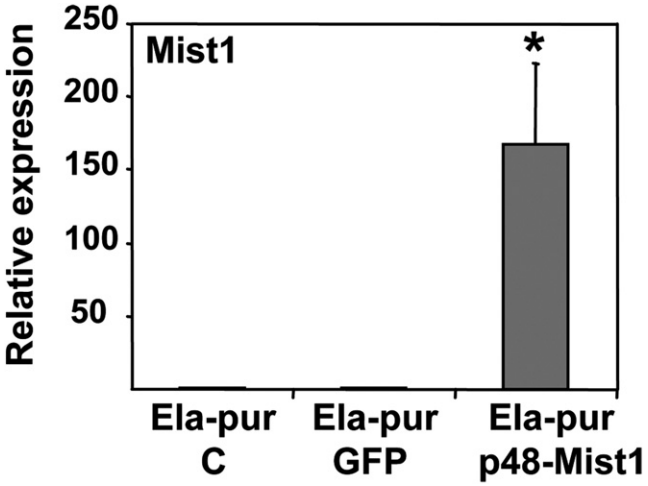


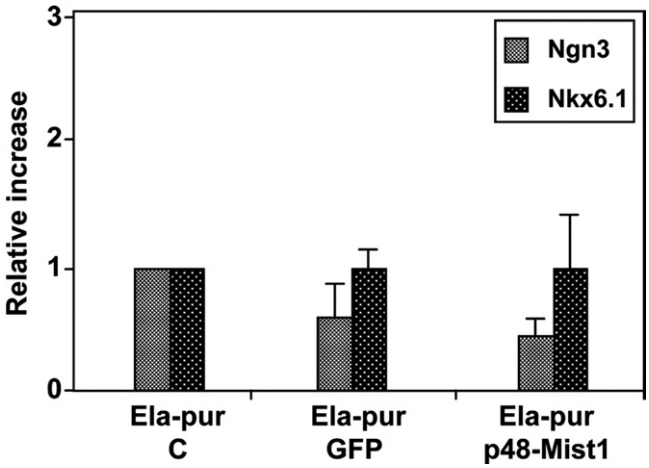
Supplementary Figure 1. Integration of the Ela promoter-puro^r-IresLacZ-pgk-Hygro^r construct into ES cell genomic DNA (*lanes 1–4*). Primers are located (*bold*) as indicated in Figure 2*A*. (−), negative control using DNA from CGR8 cells; (+), positive control.



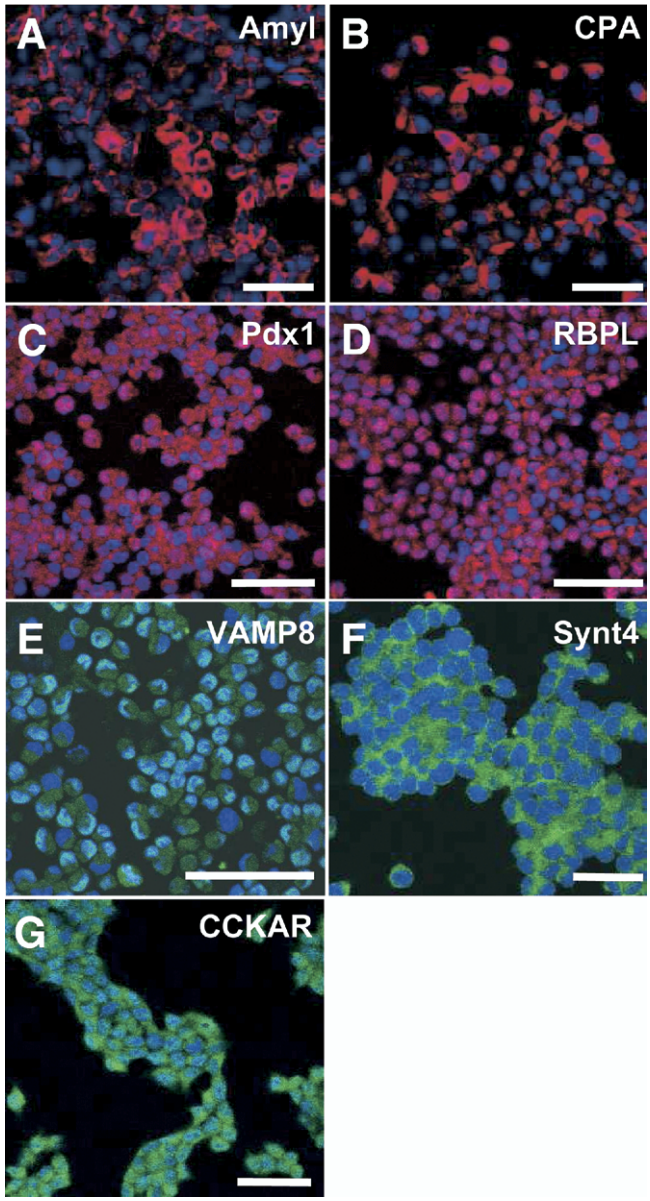
Supplementary Figure 2. Adenoviral transduction in Ela-pur ES cells. Cells were infected during the (*A–D*) differentiation step or the (*E–H*) selection process with (*A* and *E*) Adp48, (*B* and *F*) AdMist1, or (*C*, *D*, *F*, and *G*) AdGFP. The expression of the transgenes was analyzed by immunocytochemistry 2 days after infection (P2 and P23, respectively) using (*A* and *E*) rabbit anti-Ptf1a and (*B* and *F*) anti-Mist1 antibodies or by (*C* and *G*) confocal microscopy. P2 and P23 refer to the number of days in culture after EB plating. Contrast phase images are provided to indicate the cell confluency in each field. *Scale bars*: 50 μm.



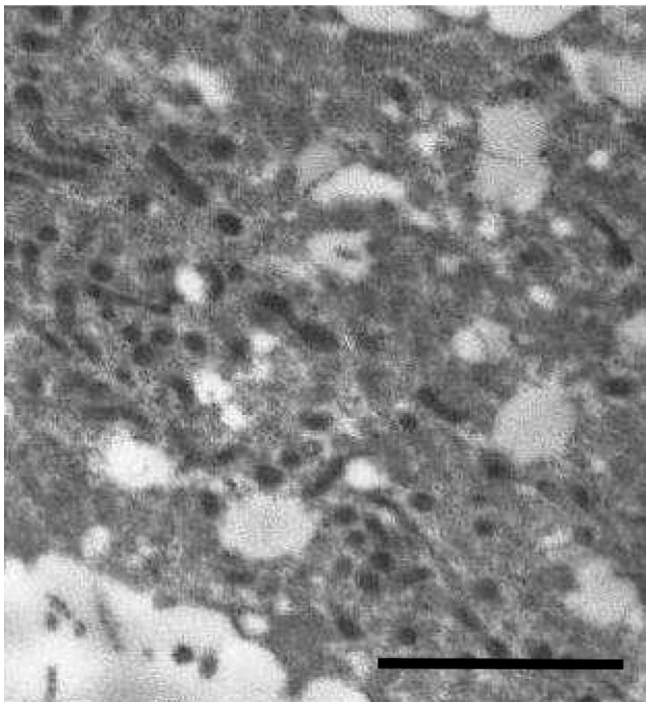
Supplementary Figure 3. Quantitative RT-PCR for the expression of endogenous and ectopic Mist1 in selected Ela-pur cells in various differentiation conditions. *Histograms* show the relative expression levels normalized to the loading control Hprt. *Error bars* indicate the standard deviations of 3 experiments. $^{*}P < .01$ compared with Ela-pur^{C}.



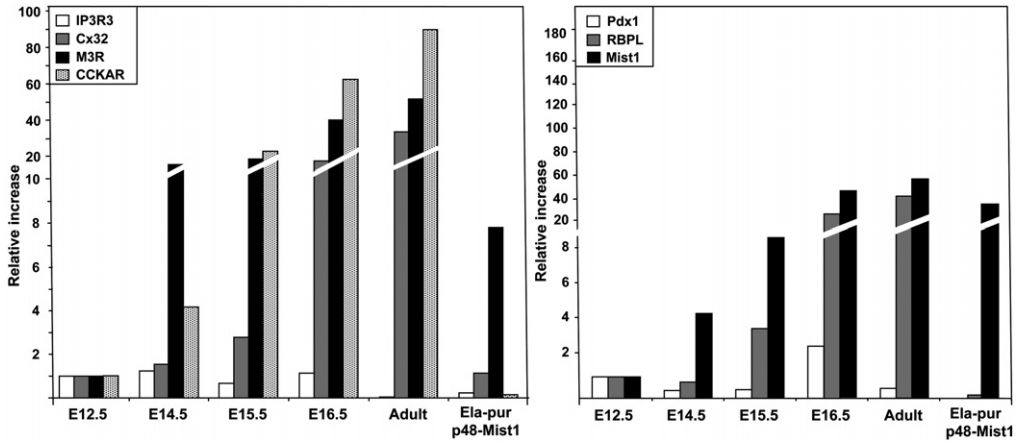
Supplementary Figure 4. qRT-PCR for the expression of pancreatic endocrine transcription factors in selected Ela-pur cells in various differentiation conditions. *Histograms* show the relative expression levels normalized to the loading control Hprt. *Error bars* indicate the standard deviations of 4 experiments.



Supplementary Figure 5. Immunofluorescence of AR42J rat acinar tumor cells for the expression of the same markers studied on Ela-pur$^{p48\text{-}Mist1}$ cells. Confocal images of AR42J cells immunostained for (*A*) Amyl, (*B*) CPA, (*C*) Pdx1, (*D*) RBPL, (*E*) VAMP8, (*F*) syntaxin 4, and (*G*) CCKAR. Nuclei were labeled with Tropo-3 iodide (blue). *Scale bar*: 50 μm.



Supplementary Figure 6. Immunogold labeling with irrelevant antibodies in Ela-pur$^{p48\text{-}Mist1}$ cells. *Scale bar*: 1.7 μm.



Supplementary Figure 7. qRT-PCR for the expression of pancreatic exocrine markers in developing and adult pancreas as well as in Ela-pur$^{p48\text{-}Mist1}$ cells. mRNAs from embryonic pancreatic rudiments, adult pancreas, and Ela-pur$^{p48\text{-}Mist1}$ cells were quantified and transcripts are expressed relative to those in the E12.5 pancreas.