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Murine ES-derived pancreatic acinar cells recapitulate features of early pancreatic differentiation

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Abstract

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 no 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 cells (ES).

Methods—Reporter ES cells (Ela-pur) were generated that stably expressed both beta-galactosidase and puromycin resistance genes under the control of the elastase I promoter. Directed differentiation was achieved by incubation with conditioned media (CM) of cultured foetal pancreatic rudiments

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No conflicts of interest exist.

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and adenoviral-mediated co-expression of p48/Ptf1a and Mist1, two bHLH 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 exhibited 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 for the first time to generate almost pure acinar-like cells from ES cells. This is the first normal cell-based model allowing the study of the acinar differentiation program *in vitro*.

Introduction

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 (M3) 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 (i.e. ZG). Because of their tumor origin, their study is also of questionable physiological 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 in order to better interfere with their loss of differentiation properties. For instance, it is generally considered that genes coding for digestive enzymes are activated simultaneously and that their expression is under identical regulatory control mechanisms, yet conventional RT-PCR data support the notion that these genes are sequentially regulated (7). Because non-quantitative RT-PCR has technical limitations, the relative levels of expression of the genes coding for acinar enzymes at different developmental stages remains to be analysed. This is important as *in vivo* tracing experiments evaluating the contribution of acinar cells to other pancreatic cell types are currently carried out 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 E10. Cells with acinar features are distinguished at E14, ZG 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 (bHLH) proteins p48/Ptf1a and Mist1, and RBPL. Ptf1a mRNA is first detected around E9.5 in pancreatic primordia and, in the adult, becomes restricted to acinar cells. Ptf1a was originally described as part of the heterotrimeric transcription complex PTF1 involved in digestive enzyme gene expression (11;12). Ptf1a is also 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 only detected 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 cells, derived from the inner mass of pre-implantation embryos, can differentiate into cells of the three 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 at generating ES cells having activated a pancreatic acinar differentiation program in order to apply this knowledge to study exocrine pancreatic diseases. Our strategy relies 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 to isolate for the first time 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 cDNA into pKS. An IresLacZ-mouse phosphoglyceratekinase (pgk) 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 μ F) using a Bio-Rad gene pulser and selected using 200 μ g/ml hygromycin to establish Ela-pur-IresLacZ clones (Ela-pur). 266-6 and AR42J pancreatic acinar tumor cells, 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-CMV 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 SI.

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% FBS without LIF. After 7 days, 30–50 EB were plated in gelatin-coated 6-well culture dishes for 7 additional days in 10% FBS supplemented medium (Differentiation step). In some conditions, 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 two days. In addition, cells were infected 36 hours after EB plating with adenoviruses as described in SI. For ES-Ela-pur selection, differentiated cultures were maintained in differentiation medium plus puromycin (0.8 μ g/ml) (Calbiochem, Darmstadt, Germany). After two 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 SI. Expression analyses were carried out by RT-PCR. Functional assays included bromodeoxyuridine incorporation, cytosolic Ca^{2+} measurements and Amyl secretion (see SI).

Statistics

Statistical differences were analyzed by the student's *t* test.

Results

Digestive enzyme gene expression is not activated as a single regulatory module during pancreatic development

Ninety per cent 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 find that acinar gene expression does not follow a single pattern (Fig. 1A). After an increase in levels of all digestive enzyme mRNAs at E14.5, distinct patterns were identified (Fig. 1A). This diversity is better reflected by comparing changes in mRNA levels of chymotrypsinogen B (ChymoB), α amylase (Amyl), and Ela1 with those of carboxypeptidase A1 (CPA). We chose CPA as reference because it is the earliest enzyme transcript detected during development (23). The CPA/ChymoB ratio remained essentially unchanged from E15.5 to adulthood (Fig. 1B), indicating similar regulation. By contrast, the CPA/Amyl and CPA/Ela1 ratios (Fig. 1B) 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 to post-natal day 2 (Fig. 1A–B). 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 an PGK-Hygro unit for selection of transfectants (Fig. 2A). Four hygromycin-resistant clones (Ela-pur) of undifferentiated ES cells screened by PCR to verify transgene integration [see supporting information (SI) (Fig. 8)] showed similar growth properties as parental cells and displayed similar expression profiles upon differentiation (not shown). Because Ela1 is already expressed at E12 of development (Fig. 1), this system will be useful to trace activation of acinar gene expression.

Differential regulation of digestive enzyme gene expression by Ptf1a and Mist1 in differentiating Ela-pur ES cell clones

Soluble signals present in conditioned medium (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, as both transcription factors are co-expressed in pancreatic cells starting at E10.5. EB from the Ela-pur clones were generated in the presence of CM and after one week allowed to adhere for 7 days (Differentiation step) (Fig. 2B). EB were additionally 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 (SI Fig. 9A–C). Control cells were generated by inducing spontaneous differentiation without additional experimental manipulation (Fig. 2B). 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 ($p=0.003$ vs. control) and, importantly, to a 2.5-fold increase in Ela1 mRNA levels (Fig. 3). Therefore, Ptf1a and Mist1 activated a gene expression pattern similar to that of early exocrine cells (Fig. 1). Because Ela1 mRNA was exclusively enhanced 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 (Fig. 2B). 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 (SI Fig. 9 E–G). Cells were analyzed after 3 weeks of selection, when isolated colonies had expanded ($n=8$) (Selection step, Fig. 2B). Undifferentiated Ela-pur ES cells did not survive puromycin selection ($n=3$). After inducing differentiation with CM and Ptf1a and Mist1 adenoviruses (referred as Ela-pur^{p48-Mist1} after selection), >95% cells were strongly β galactosidase-positive ($n=6$) (Fig. 4A). 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 (Fig. 4B–C). Highest digestive enzyme mRNA levels were achieved in Ela-pur^{p48-Mist1}. Interestingly, only CPA and ChymoB increased up to 300-fold in comparison with spontaneously differentiated and selected cells (Ela-pur^C) (Fig. 4B). The ratio of CPA/ChymoB mRNA levels was 0.43, a value similar to that in early (E12.5–E14.5) pancreatic development (Fig. 1B). Importantly, these effects were associated with an increase in endogenous Ptf1a and RBPL mRNAs but not of endogenous Mist1 (Fig. 4C). As expected, high levels of ectopic Mist1 mRNA were detected (SI Fig. 10). Pdx1, a transcription factor involved in both exocrine and endocrine differentiation was expressed - but not significantly modulated - in these conditions (Fig. 4C), 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 (Fig. 4C). Ela-pur^{p48-Mist1} cells expressed mRNAs coding for CCKA and M3 receptors as well as IP3R3, an important signaling mediator. Transcripts coding for connexin 32, a gap junctional protein required for exocrine secretion (35), were also detected. By contrast, endocrine marker levels were very low (Ngn3, Nkx6.1) (SI Fig. 11) or undetectable (insulin). CK19 and CFTR (ductal) mRNA levels were not modulated (Fig. 4C).

To assess whether physiological cytodifferentiation was engaged, expression of selected corresponding proteins was studied using immunofluorescence. Ninety-eight percent of Ela-pur^{p48-Mist1} cells expressed E-cadherin and β -catenin at cell-cell contact sites (results from 3 independent experiments), demonstrating their epithelial nature (Fig. 5A–B). Cytoplasmatic Amyl and CPA were detected in most cells ($67 \pm 3.8\%$; $80 \pm 1.54\%$, respectively) (Fig. 5C–D), as in AR42J cells (SI Fig. 12A–B). In agreement with the RT-PCR results, nuclear Pdx1 (Fig. 5E) and RBPL (Fig. 5F) were expressed. These proteins were undetectable in undifferentiated Ela-pur cells (Fig. 5C'–F'). Regarding the secretory signaling pathway and machinery, CCKAR (Fig. 5I), VAMP8 - a SNARE protein associated with ZG whose function is crucial for secretion (36) (Fig. 5G), and syntaxin 4 - a membrane protein of pancreatic acinar cells (36) - (Fig. 5H) displayed membrane (Fig. 5H, I) or cytoplasmic patterns (Fig. 5G). By contrast, Dolichos biflorus agglutinin (DBA) - which selectively recognizes ductal cells (37)

- was restricted to very few cells ($1.8 \pm 1.82\%$) that were β -galactosidase- negative (Fig. 5J). Moreover, BrdU uptake assays demonstrated that Ela-pur^{p48-Mist1} cells exhibited reduced proliferative ability as compared with other experimental conditions (Fig. 4D). In addition, cyclin D1 mRNA levels were reduced in these cells (Fig. 4E). Collectively, these data demonstrate the coordinated activation of an acinar differentiation program in Ela-pur^{p48-Mist1} cells.

Ela-pur^{p48-Mist1} cells contain zymogen granules and respond to acinar secretagogues

A hallmark of acinar cells is enzyme storage in electron-dense ZG (Fig. 6A). Ela-pur^{p48-Mist1} cells displayed 250–600 nm ZG-like vesicles (Fig. 6B) which were very scarce in spontaneously differentiated cells (Fig. 6C) 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 above). Gold immunoelectron microscopy demonstrated that these vesicles contain Amyl (Fig. 6E) and (SI Fig. 13).

To further investigate their functional properties, cells were loaded with the calcium-sensitive dye fura-2 and the intracellular Ca^{2+} response to 5 μM carbachol was monitored. Fig. 7A summarizes results from 4 experiments. Ela-pur^{p48-Mist1} cells (Fig. 7A, right) displayed a rapid and transient increase in Ca^{2+} concentration, similar to that described for primary isolated mouse acinar 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 two conditions ($P < 0.05$) while the plateau values at the end of the carbachol pulse were not different ($P > 0.05$). The percentage of carbachol-responsive cells was higher in Ela-pur^{p48-Mist1} (35%; 97/280) than in Ela-pur^C (14%; 33/328). 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* (Fig. 7B). Carbachol and CCK induced a significant increase in total Amyl activity (left); this effect was more pronounced in Ela-pur^{p48-Mist1} cells ($P < 0.05$ compared to 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 (middle), being more pronounced in Ela-pur^{p48-Mist1} cells ($P < 0.05$). 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 for the first time, 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 staining showing that nearly all cells expressed the reporter construct. Contamination by other cell types (i.e., 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 upon spontaneous differentiation indeed express Ptf1a and Mist1 though at levels that are much lower than found in cells transduced with Ptf1a and Mist1, providing evidences that regulation

of transcription factors levels is a key step during acinar differentiation. In this regard, we found that endogenous Ptf1a was also up-regulated by co-expression of Ptf1a and Mist-1, suggesting the existence of an auto-regulatory loop favouring exocrine differentiation. Indeed, such auto-regulatory circuits have been recently described during exocrine pancreatic development. Thus, RBPL is able to auto-regulate 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 as endogenous mRNA levels were not modulated (Fig 4C), probably due to the high levels achieved after gene transduction which were found quite similar to those observed at E16.5 and in adult pancreas (SI Fig. 14). Further, we demonstrated 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 has been very recently reported that the ability of RBPL to regulate the expression of the zymogen genes is not universal (24). Interestingly, it has been 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 could also 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 efficiently transduced by adenoviral transgene delivery during selection. Because Ela-pur^C cells which spontaneously differentiated exhibited an aberrant pattern of Ca²⁺ 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 Ca²⁺ signalling machinery more mature in elastase-producing cells, as has been proposed *in vivo*. Also, the percentage of cells exhibiting zymogen granule-like vesicles was higher in Ela-pur^{p48-Mist1} cells than in any other experimental condition, in agreement with the fact that the number of zymogen granules is reduced in Mist1 knock-out mice (19). Overall, we show that the directed-differentiated cellular response to Ptf1a and Mist1 is similar of 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 de-differentiation 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 upon mature acinar cell isolation and *in vitro* culture. The signalling pathways proposed to be activated under these conditions are mediated by Notch and EGF 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 signalling pathway in the culture conditions tested here (not shown) and the expression of Pdx1, which is induced during the acinar-ductal switch, was also 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 de/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 (i.e., 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 are currently underway in our laboratories.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

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
M3	muscarinic 3
ZG	zymogen granules

References

1. Williams JA. Regulation of pancreatic acinar cell function. *Curr Opin Gastroenterol* 2006;22:498–504. [PubMed: 16891880]
2. Means AL, Meszoely IM, Suzuki K, Miyamoto Y, Rustgi AK, Coffey RJ, Wright CVE, Stoffers DA, Leach SD. Pancreatic epithelial plasticity mediated by acinar cell transdifferentiation and generation of nestin-positive intermediates. *Development* 2005;132:3767–3776. [PubMed: 16020518]
3. Miyamoto Y, Maitra A, Ghosh B, Zechner U, Argani P, Iacobuzio-Donahue CA, Sriuranpong V, Iso T, Meszoely IM, Wolfe MS, Hruban RH, Ball DW, Schmid RM, Leach SD. Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis. *Cancer Cell* 2003;3:565–576. [PubMed: 12842085]
4. Sandgren EP, Luetke NC, Palmiter RD, Brinster RL, Lee DC. Overexpression of TGF alpha in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. *Cell* 1990;61:1121–1135. [PubMed: 1693546]
5. Schmid RM. Acinar-to-ductal metaplasia in pancreatic cancer development. *J Clin Invest* 2002;109:1403–1404. [PubMed: 12045253]
6. Zhu L, Shi G, Schmidt CM, Hruban RH, Konieczny SF. Acinar cells contribute to the molecular heterogeneity of pancreatic intraepithelial neoplasia. *Am J Pathol* 2007;171:263–273. [PubMed: 17591971]
7. Gittes GK, Rutter WJ. Onset of cell-specific gene expression in the developing mouse pancreas. *Proc Natl Acad Sci U S A* 1992;89:1128–1132. [PubMed: 1371010]
8. Kim SK, MacDonald RJ. Signaling and transcriptional control of pancreatic organogenesis. *Curr Opin Genet Dev* 2002;12:540–547. [PubMed: 12200159]
9. Jensen J. Gene regulatory factors in pancreatic development. *Dev Dyn* 2004;229:176–200. [PubMed: 14699589]
10. Murtaugh LC. Pancreas and beta-cell development: from the actual to the possible. *Development* 2007;134:427–438. [PubMed: 17185316]
11. Rose SD, Swift GH, Peyton MJ, Hammer RE, MacDonald RJ. The role of PTF1-P48 in pancreatic acinar gene expression. *J Biol Chem* 2001;276:44018–44026. [PubMed: 11562365]
12. Petrucco S, Wellauer PK, Hagenbuchle O. The DNA-binding activity of transcription factor PTF1 parallels the synthesis of pancreas-specific mRNAs during mouse development. *Mol Cell Biol* 1990;10:254–264. [PubMed: 2294404]
13. Krapp A, Knofler M, Ledermann B, Burki K, Berney C, Zoerkler N, Hagenbuchle O, Wellauer PK. The bHLH protein PTF1-p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas. *Genes Dev* 1998;12:3752–3763. [PubMed: 9851981]
14. Kawaguchi Y, Cooper B, Gannon M, Ray M, MacDonald RJ, Wright CV. The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. *Nat Genet* 2002;32:128–134. [PubMed: 12185368]

15. Lin JW, Biankin AV, Horb ME, Ghosh B, Prasad NB, Yee NS, Park MA, Leach SD. Differential requirement for *ptf1a* in endocrine and exocrine lineages of developing zebrafish pancreas. *Dev Biol* 2004;270:474–486. [PubMed: 15183727]
16. Afelik S, Chen YL, Pieler T. Combined ectopic expression of *Pdx1* and *Ptf1a/p48* results in the stable conversion of posterior endoderm into endocrine and exocrine pancreatic tissue. *Genes Dev* 2006;20:1441–1446. [PubMed: 16751182]
17. Jarikji ZH, Vanamala S, Beck CW, Wright CVE, Leach SD, Horb ME. Differential ability of *Ptf1a* and *Ptf1a-VP16* to convert stomach, duodenum and liver to pancreas. *Dev Biol* 2007;304:786–799. [PubMed: 17320068]
18. Fukuda A, Kawaguchi Y, Furuyama K, Kodama S, Horiguchi M, Kuhara T, Koizumi M, Boyer DF, Fujimoto K, Doi R, Kageyama R, Wright CVE, Chiba T. Ectopic pancreas formation in *Hes1*-knockout mice reveals plasticity of endodermal progenitors of the gut, bile duct, and pancreas. *J Clin Invest* 2006;116:1484–1493. [PubMed: 16710472]
19. Johnson CL, Kowalik AS, Rajakumar N, Pin CL. *Mist1* is necessary for the establishment of granule organization in serous exocrine cells of the gastrointestinal tract. *Mech Dev* 2004;121:261–272. [PubMed: 15003629]
20. Pin CL, Rukstalis JM, Johnson C, Konieczny SF. The bHLH transcription factor *Mist1* is required to maintain exocrine pancreas cell organization and acinar cell identity. *J Cell Biol* 2001;155:519–30. [PubMed: 11696558]
21. Zhu L, Tran T, Rukstalis JM, Sun P, Damsz B, Konieczny SF. Inhibition of *Mist1* homodimer formation induces pancreatic acinar-to-ductal metaplasia. *Mol Cell Biol* 2004;24:2673–2681. [PubMed: 15024058]
22. Beres TM, Masui T, Swift GH, Shi L, Henke RM, MacDonald RJ. PTF1 is an organ-specific and notch-independent basic helix-loop-helix complex containing the mammalian suppressor of hairless (RBP-J) or its paralogue, RBP-L. *Mol Cell Biol* 2006;26:117–130. [PubMed: 16354684]
23. Zhou Q, Law AC, Rajagopal J, Anderson WJ, Gray PA, Melton DA. A multipotent progenitor domain guides pancreatic organogenesis. *Dev Cell* 2007;13:103–114. [PubMed: 17609113]
24. Masui T, Long Q, Beres TM, Magnuson MA, MacDonald RJ. Early pancreatic development requires the vertebrate Suppressor of Hairless (RBPJ) in the PTF1 bHLH complex. *Genes Dev* 2007;21:2629–2643. [PubMed: 17938243]
25. Skoudy A, Rovira M, Savatier P, Martin F, Leon-Quinto T, Soria B, Real FX. Transforming growth factor (TGF) β , fibroblast growth factor (FGF) and retinoid signalling pathways promote pancreatic exocrine gene expression in mouse embryonic stem cells. *Biochem J* 2004;379:749–756. [PubMed: 14733613]
26. Wobus AM, Boheler KR. Embryonic stem cells: prospects for developmental biology and cell therapy. *Physiol Rev* 2005;85:635–678. [PubMed: 15788707]
27. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol* 2006;24:1392–1401. [PubMed: 17053790]
28. MacDonald RJ, Swift GH. Analysis of transcriptional regulatory regions in vivo. *Int J Dev Biol* 1998;42:983–994. [PubMed: 9853829]
29. Rovira M, Jane-Valbuena J, Marchand M, Savatier P, Real FX, Skoudy A. Viral-mediated coexpression of *Pdx1* and *p48* regulates exocrine pancreatic differentiation in mouse ES cells. *Cloning Stem Cells* 2007;9:327–338. [PubMed: 17907943]
30. Rodolosse A, Chalaux E, Adell T, Hagege H, Skoudy A, Real FX. PTF1 α /p48 transcription factor couples proliferation and differentiation in the exocrine pancreas and cell proliferation. *Gastroenterology* 2004;127:937–949. [PubMed: 15362048]
31. Pin CL, Lemerrier C, Konieczny SF. Cloning of the murine *Mist1* gene and assignment to mouse chromosome band 5G2-5G3. *Cytogenet Cell Genet* 1999;86:219–222. [PubMed: 10575209]
32. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A* 1998;95:2509–2514. [PubMed: 9482916]
33. Heller RS, Stoffers DA, Bock T, Svenstrup K, Jensen J, Horn T, Miller CP, Habener JF, Madsen OD, Serup P. Improved glucose tolerance and acinar dysmorphogenesis by targeted expression of

- transcription factor PDX-1 to the exocrine pancreas. *Diabetes* 2001;50:1553–1561. [PubMed: 11423476]
34. Miyatsuka T, Kaneto H, Shiraiwa T, Matsuoka TA, Yamamoto K, Kato K, Nakamura Y, Akira S, Takeda K, Kajimoto Y, Yamasaki Y, Sandgren EP, Kawaguchi Y, Wright CV, Fujitani Y. Persistent expression of PDX-1 in the pancreas causes acinar-to-ductal metaplasia through Stat3 activation. *Genes Dev* 2006;20:1435–1440. [PubMed: 16751181]
 35. Chanson M, Fanjul M, Bosco D, Nelles E, Suter S, Willecke K, Meda P. Enhanced secretion of amylase from exocrine pancreas of connexin32-deficient mice. *J Cell Biol* 1998;141:1267–1275. [PubMed: 9606217]
 36. Wang CC, Ng CP, Lu L, Atlashkin V, Zhang W, Seet LF, Hong WJ. A role of VAMP8/endobrevin in regulated exocytosis of pancreatic acinar cells. *Dev Cell* 2004;7:359–371. [PubMed: 15363411]
 37. Stanger BZ, Stiles B, Lauwers GY, Bardeesy N, Mendoza M, Wang Y, Greenwood A, Cheng KH, McLaughlin M, Brown D, DePinho RA, Wu H, Melton DA, Dor Y. Pten constrains centroacinar cell expansion and malignant transformation in the pancreas. *Cancer Cell* 2005;8:185–195. [PubMed: 16169464]
 38. Luo X, Shin DM, Wang XH, Konieczny SF, Muallem S. Aberrant localization of intracellular organelles, Ca²⁺ signaling, and exocytosis in Mist1 null mice. *J Biol Chem* 2005;280:12668–12675. [PubMed: 15665001]
 39. Vila MR, Lloreta J, Real FX. Normal human pancreas cultures display functional ductal characteristics. *Lab Invest* 1994;71:423–431. [PubMed: 7523764]
 40. Rooman I, De Medts N, Baeyens L, Lardon J, De Breuck S, Heimberg H, Bouwens L. Expression of the notch signaling pathway and effect on exocrine cell proliferation in adult rat pancreas. *Am J Pathol* 2006;169:1206–1214. [PubMed: 17003479]
 41. Delisle RC, Logsdon CD. Pancreatic acinar-cells in culture - Expression of acinar and ductal antigens in a growth-related manner. *Eur J Clin Invest* 1990;51:64–75.
 42. Sphyris N, Logsdon CD, Harrison DJ. Improved retention of zymogen granules in cultured murine pancreatic acinar cells and induction of acinar-ductal transdifferentiation in vitro. *Pancreas* 2005;30:148–157. [PubMed: 15714137]
 43. Hruban RH, Adsay NV, Albores-Saavedra J, Anver MR, Biankin AV, Boivin GP, Furth EE, Furukawa T, Klein A, Klimstra DS, Kloppel G, Lauwers GY, Longnecker DS, Luttges J, Maitra A, Offerhaus GJ, Perez-Gallego L, Redston M, Tuveson DA. Pathology of genetically engineered mouse models of pancreatic exocrine cancer: consensus report and recommendations. *Cancer Res* 2006;66:95–106. [PubMed: 16397221]
 44. Hingorani SR, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, Ross S, Conrads TP, Veenstra TD, Hitt BA, Kawaguchi Y, Johann D, Liotta LA, Crawford HC, Putt ME, Jacks T, Wright CV, Hruban RH, Lowy AM, Tuveson DA. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell* 2003;4:437–450. [PubMed: 14706336]
 45. Guerra C, Schuhmacher AJ, Canamero M, Grippo PJ, Verdaguer L, Perez-Gallego L, Dubus P, Sandgren EP, Barbacid M. Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by k-Ras oncogenes in adult mice. *Cancer Cell* 2007;11:291–302. [PubMed: 17349585]
 46. Sandgren EP, Quaife CJ, Paulovich AG, Palmiter RD, Brinster RL. Pancreatic tumor pathogenesis reflects the causative genetic lesion. *Proc Natl Acad Sci U S A* 1991;88:93–97. [PubMed: 1986386]

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-pgk-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 save of simplicity, this step is 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 is designated as “selection”, although differentiation related events can take place during this phase.

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 Fig. 2B. After EB adhesion, cells were infected - or not - with AdGFP, Adp48, AdMist1 or with a combination of both Adp48 and AdMist1. Error bars indicate the standard deviations of two independent experiments.

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 μ m. (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 four experiments in (B) and of two-three experiments in (C); * $P < 0.05$; ** $P < 0.01$, compared to Ela-pur^C cells. (D) BrdU incorporation in the differentiation conditions analysed by FACS ($n = 3$). * $P < 0.05$; ** $P < 0.01$, compared to Ela-pur^C cells. (E) Cyclin D1 expression by q-PCR analyzed as in (C).

Figure 5.

Immunofluorescence analysis of Ela-pur^{p48-Mist1} cells. Confocal images of cells immunostained for E-cadherin (A), β-catenin (B), Amyl (C), CPA (D), Pdx1 (E), RBPL (F), VAMP8 (G), syntaxin 4 (H) and CCKAR (I). 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-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). The box in (J) shows a brown cell stained for DBA in a double staining with X-Gal. Scale bars: A-I, 50 μm; J, 100 μm. Figure 12 in SI shows immunostaining of AR42J acinar cells with the same antibodies, for comparison.

Figure 6.

Immuno-electron analysis of Ela-pur^{p48-Mist1} cells. Electron micrographs illustrating electron-dense vesicles in (A) murine pancreas (inset) and (B) Ela-pur^{p48-Mist1} cells (arrows). (C) Histograms of data from two 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) Ela-pur^{p48-Mist1}. Scale bars: B, 1 μ m; D, 0.6 μ m; E, 1.7 μ m.

Figure 7.

Carbachol-evoked Ca^{2+} signalling and exocytosis in Ela-pur selected cells. (A) Cytosolic Ca^{2+} signals in Fura-2-loaded cells stimulated with 5 μ M carbachol for 2.5 minutes. At the indicated times, the secretagogue was superfused and removed. Ela-pur^C, n = 33; Ela-pur^{p48-Mist1}, n = 97. (B) Selected cells were stimulated for 30 min 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 two experiments performed in triplicate.