BASIC AND TRANSLATIONAL—PANCREAS

Let-7b and miR-495 Stimulate Differentiation and Prevent Metaplasia of Pancreatic Acinar Cells by Repressing HNF6

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BACKGROUND & AIMS: Diseases of the exocrine pancreas are often associated with perturbed differentiation of acinar cells. MicroRNAs (miRNAs) regulate pancreas development, yet little is known about their contribution to acinar cell differentiation. We aimed to identify miRNAs that promote and control the maintenance of acinar differentiation. **METHODS:** We studied mice with pancreas- or acinar-specific inactivation of Dicer (*Foxa3-Cre/Dicer*$^{loxP/-}$ mice), combined (or not) with inactivation of hepatocyte nuclear factor (HNF) 6 (*Foxa3-Cre/Dicer*$^{loxP/-}$*/Hnf6−/−* mice). The role of specific miRNAs in acinar differentiation was investigated by transfecting cultured cells with miRNA mimics or inhibitors. Pancreatitis-induced metaplasia was investigated in mice after administration of cerulein. **RESULTS:** Inhibition of miRNA synthesis in acini by inactivation of Dicer and pancreatitis-induced metaplasia were associated with repression of acinar differentiation and with induction of HNF6 and hepatic genes. The phenotype of Dicer-deficient acini depends on the induction of HNF6; overexpression of this factor in developing acinar cells is sufficient to repress acinar differentiation and to induce hepatic genes. Let-7b and miR-495 repress HNF6 and are expressed in developing acini. Their expression is inhibited in Dicer-deficient acini, as well as in pancreatitis-induced metaplasia. In addition, inhibiting let-7b and miR-495 in acinar cells results in similar effects to those found in Dicer-deficient acini and metaplastic cells, namely induction of HNF6 and hepatic genes and repression of acinar differentiation. **CONCLUSIONS: Let-7b, miR-495, and their targets constitute a gene network that is required to establish and maintain pancreatic acinar cell differentiation. Additional studies of this network will increase our understanding of pancreatic diseases.**

Keywords: Mouse Model; Pancreatic Development; Proliferation; Gene Regulation.

The role of microRNAs (miRNAs) in pancreas development was initially characterized by phenotyping mice with pancreas-specific inactivation of Dicer, an enzyme essential for miRNA maturation. Depletion of miRNAs at the onset of pancreas development led to pancreatic hypoplasia and abnormal development of all epithelial cell lineages.[1] In particular, reduction in the number of insulin-producing β cells was striking, and expression of the proendocrine factor Neurogenin 3 was reduced. In another study, partial depletion of miRNAs resulting from the use of a hypomorphic allele of *Dicer* allowed normal pancreas development, but was associated with postnatal endocrine regeneration and irregular shape of some acini.[2] In addition, a number of individual miRNAs were shown to regulate endocrine cell development and function.[3,4] However, how miRNAs impact on acinar cell differentiation remains unknown.

Differentiation of pancreatic progenitors to acinar cells starts around embryonic day (E) 13.5−14.5. This stage coincides with the coexpression of early acinar proteins, such as pancreas-specific transcription factor subunit 1a (Ptf1a/p48), carboxypeptidase A (Cpa), and amylase (Amy; expression of Cpa slightly precedes that of Amy) at the extremities of branched tubules. Subsequently, acinar differentiation proceeds until birth by morphogenesis of acini, and acquisition of the full set of acinar-specific functions.[5] Ptf1a, recombining binding protein/suppressor of hairless (Rbpj), and Mist1 are key transcriptional regulators of acinar development.[6–8] At the earliest stage of pancreas development, Ptf1a forms a complex with Rbpj and controls the development of pancreatic progenitors from foregut endoderm.[9,10] When acinar cells differentiate, Rbpjl progressively replaces Rbpj in the complex, which is necessary for activation of acinar genes.[11,12] The importance of Rbpjl in acinar cell differentiation and maturation is underlined by the phenotype of Rbpjl$^{-/-}$ mice that show a strong reduction in the expression of genes coding for acinar-specific functions, and ectopic expression of hepatic genes.[11] Finally, Mist1 is required for acinar cell organization, and its absence leads to a partial switch toward a ductal phenotype.[7,8,13] To our knowledge, no link was

Abbreviations used in this paper: Amy, amylase; Apo, apolipoprotein; Cel, carboxyl-ester lipase; CK19, cytokeratin 19; Cpa, carboxypeptidase A; E, embryonic day; GFP, green fluorescent protein; HNF, hepatocyte nuclear factor; miRNA, microRNA; P, postnatal day; Prss1, trypsin 1; Ptf1a, pancreas-specific transcription factor subunit 1a; Rbpj, recombining binding protein suppressor of hairless; si, small interfering; Try4, trypsin4.

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found between miRNAs and acinar-specific transcription factors.

Several in vitro and in vivo models revealed that acinar cells display an unexpected plasticity in disease conditions.[5] The cells switch off their own differentiation program and acquire alternative gene expression signatures that are typical of ductal cells (acinar-to-ductal metaplasia) or of hepatocytes (acinar-to-hepatic metaplasia).[14] Pancreatic adenocarcinoma is thought to arise from acinar cells,[15] and metaplasia is considered as an initiating event in cancerogenesis. Several studies have shown that miRNA expression is modified in pancreatic metaplasia and pancreatic adenocarcinoma.[16] However, to our knowledge, no functional link between miRNAs and perturbed acinar differentiation has been established. In the present work, we found that let-7b and miR-495 repress HNF6, a transcription factor whose increased expression in acinar cells is sufficient to repress Mist1 and Rbpjl, and to inhibit acinar differentiation while promoting ectopic liver gene expression. Reducing expression of let-7b and miR-495 recapitulates the differentiation anomalies found in pancreatitis-associated metaplasia. This work uncovers a gene network that is critical to establish and maintain acinar homeostasis.

Materials and Methods

Mice

Mice received humane care according to the criteria of the National Academy of Sciences and all experiments were performed with approval of the institution's welfare committee. *Hnf6*$^{-/-}$, *Dicer*$^{loxP/loxP}$, *PGK-Cre*, *Foxa3-Cre*, *Rosa26R-EYFP*, and *Elastase-Cre* mice are described.[17–22] *Dicer*$^{wt/loxP}$ and PGK-Cre mice were mated to obtain *Dicer*$^{wt/-}$ mice. *Dicer*$^{loxP/loxP}$ and *Foxa3-Cre/Dicer*$^{wt/-}$ or *Ela-Cre/Dicer*$^{wt/-}$ mice were crossed to obtain *Foxa3-Cre/Dicer*$^{loxP/-}$ or *Ela-Cre/Dicer*$^{loxP/-}$ embryos.

Cerulein Treatment

Acute pancreatitis was induced by 8 hourly intraperitoneal injections of cerulein (75 μg/kg; Sigma Aldrich, Bornem, Belgium) in Ela-Cre/Rosa26R-EYFP mice (30 g, 6–8 weeks of age).[23] Cerulein was administered on 2 consecutive days. The second day of injections was considered day 0. Pancreata were collected after 2 days. Experimental conditions included 3 phosphate-buffered saline–injected control and 3 cerulein-injected mice.

Real-Time Quantitative Polymerase Chain Reaction

RNA was extracted from pancreata or cultured cells with TriPure reagent (Roche, Basel, Switzerland). Real-time quantitative polymerase chain reaction quantification of miRNAs was performed as described previously,[24,25] with modifications (Supplementary Material).

Immunohistochemistry and Immunofluorescence

Pancreata were fixed for 6 hours in 4% paraformaldehyde at 4°C before embedding in paraffin or gelatin. Serial sections (9 μm) were analyzed (Supplementary Material).

Ex Vivo Culture and Adenovirus Infection

E15.5 pancreata were collected from CD1 mice and dissociated for 10 minutes at 37°C in 1 mL enzyme mix (collagenase P 0.25 mg/mL; collagenase IV, 0.35 mg/mL; dispase, 1 mg/mL; Sigma). When clusters of 15–20 acinar cells were obtained, the reaction was stopped by adding 10 mL RPMI supplemented with 10% of fetal calf serum. Acinar clusters sedimented rapidly, enabling elimination of the supernatant, which contains mesenchymal cells, duct, and vessel fragments. After centrifugation and washing, cell clusters were cultured on dishes with Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, L-glutamine, and antibiotics. After 6 hours, residual fibroblasts adhered to the culture dishes, while floating acinar cell–enriched clusters were transferred to Petri dishes. Cells were infected with adenovirus-expressing HNF6 and green fluorescent protein (GFP) (Ad-HNF6),[26] or GFP alone (Ad-GFP) (Vector BioLabs, Philadelphia, PA) at a multiplicity of infection of 100 in 200 μL Dulbecco's modified Eagle medium. Cells were grown in suspension for 72 hours and washed before RNA extraction.

Chromatin Immunoprecipitation

Samples for chromatin immunoprecipitation were collected from acinar cell–enriched culture infected with Ad-HNF6 or Ad-GFP. Proteins were cross linked to DNA using 1% formaldehyde in serum-free medium for 10 minutes at room temperature. Cross linking was stopped by adding glycine at a final concentration of 125 mM. Cells were washed in ice-cold phosphate-buffered saline treated with sodium dodecyl sulfate lysis buffer (Millipore, Waterven, Belgium) and protease inhibitors (Sigma) for 10 minutes on ice. Samples were sonicated for 10 minutes (30 seconds/30 seconds off) at ultrasonic wave output power 320 W in Bioruptor (Diagenode, Liège, Belgium). Chromatin was sedimented at 14,000 rpm for 10 minutes. Chromatin immunoprecipitation was performed using the OneDay ChIP Kit (Diagenode). Samples were incubated with nonimmune IgG (negative control) or with anti-HNF6 antibody (SC-13050; Santa Cruz Biotechnology, Santa Cruz, CA). Precipitated DNA was analyzed by real-time quantitative polymerase chain reaction and normalized for input. Values obtained with AdHNF6 or AdGFP in the presence of IgG were arbitrarily set to 1 and served as a reference to calculate the fold-enrichment in the presence of anti-HNF6 antibody. A negative (HNF6 nonbinding) control region was selected in the mouse *HNF1β* promoter. Primer sequences are described in Supplementary Table 1.

Fluorescence-Activated Cell Sorting

Acinar cells were separated from non-acinar cells by cell sorting using EYFP fluorescence. Five to 10 pancreata from *Elastase-Cre/ROSA26R-EYFP* embryos were microdissected at E14.5, E17.5, and postnatal day (P) 0. Pancreata were dissociated in 500 μL trypsin 0.05% (Invitrogen, Carlsbad, CA) at 37°C and stopped with 500 μL RPMI + 10% serum. The cell suspension (1 mL) was filtered through a 40-μm nylon filter (Cell Strainer, BD Biosciences, Erembodegem, Belgium), cell aggregates retained on the filter were washed once with 1 mL RPMI, and the total filtrate (2 mL) containing the single-cell suspension was sorted on a FACS Aria III (BD Biosciences). A 85-μm nozzle was used with default conditions of sheath pressure (45 psi) and drop drive frequency (47 kHz). Sample injection chamber and collection tubes were maintained at 4°C.

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