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ScienceDirect
Developmental Biology 311 (2007) 603–612
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

Genomes & Developmental Control

MicroRNA profiling of developing and regenerating pancreas reveal post-transcriptional regulation of neurogenin3

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Received for publication 9 May 2007; revised 21 August 2007; accepted 6 September 2007
Available online 18 September 2007

Abstract

The mammalian pancreas is known to show a remarkable degree of regenerative ability. Several studies until now have demonstrated that the mammalian pancreas can regenerate in normal as well as diabetic conditions. These studies illustrate that pancreatic transcription factors that are seen to be expressed in a temporal fashion during development are re-expressed during regeneration. The only known exception to this is Neurogenin3 (NGN3). Though NGN3 protein, which marks all the pro-endocrine cells during development, is not seen during mouse pancreas regeneration, functional neo-islets are generated by 4 weeks after 70% pancreatectomy. We observed that pancreatic transcription factors upstream of ngn3 showed similar gene expression patterns during development and regeneration. However, gene transcripts of transcription factors immediately downstream of ngn3 (neuroD and nkx2.2) did not show such similarities in expression. Since NGN3 protein was not detected at any time point during regeneration, we reasoned that post-transcriptional silencing of ngn3 by microRNAs may be a possible mechanism. We carried out microRNA analysis of 283 known and validated mouse microRNAs during different stages of pancreatic development and regeneration and identified that 4 microRNAs; miR-15a, miR-15b, miR-16 and miR-195, which can potentially bind to ngn3 transcript, are expressed at least 200-fold higher in the regenerating mouse pancreas as compared to embryonic day (e) 10.5 or e 16.5 developing mouse pancreas. Inhibition of these miRNAs in regenerating pancreatic cells using anti-sense miRNA-specific inhibitors, induces expression of NGN3 and its downstream players: neuroD and nkx2.2. Similarly, overexpression of miRNAs targeting ngn3 during pancreas development shows reduction in the number of hormone-producing cells. It appears that during pancreatic regeneration in mice, increased expression of these microRNAs allows endocrine regeneration via an alternate pathway that does not involve NGN3 protein. Our studies on microRNA profiling of developing and regenerating pancreas provide us with better understanding of mechanisms that regulate post-natal islet neogenesis.

Keywords: Pancreas development; Regeneration; Pancreatectomy; microRNA; Neurogenin3

Introduction

Failure in maintenance of pancreatic β-cell mass is recognized to be a major player in pathogenesis of type 1 and type 2 diabetes mellitus. Insulin replacement therapy, achieved by transplantation of cadaveric pancreatic insulin-producing cells, has been demonstrated with some success (Harlan and Rother, 2004; Shapiro et al., 2000). However, an alternative approach to islet transplantation is stimulation of endogenous pancreatic β-cell regeneration. Regeneration of endocrine as well as exocrine pancreas has been shown to occur in mice under non-diabetic as well as diabetic conditions (Bonner-Weir et al., 1993; Hardikar et al., 1999). Understanding the mechanisms involved in β-cell development and regeneration will enlighten therapeutic efforts to augment the number of functional β-cells in patients with diabetes.

Endocrine pancreas development in mice begins at the junction of foregut and midgut as dorsal and ventral budding of the gut tube (Schwitzgebel et al., 2000; Wells, 2003). As the gut

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0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.09.008

tube rotates during development, dorsal and ventral buds fuse with each other to form the definitive pancreas and endocrine cells are generated from duct-like structures in the developing pancreas. At embryonic day (e) 14, the termini of these duct-like structures form acini and differentiate into exocrine cells. At this time ("secondary transition"), there is a huge increase in the transcript level as well as number of insulin producing cells (Sander et al., 2000; Wang et al., 2005). During mouse pancreas development, cell fate is determined by a very nicely regulated and synchronized spatio-temporal expression of transcription factors, of which Neurogenin3 (NGN3), a bHLH transcription factor, marks pancreatic endocrine progenitor cells, as confirmed by lineage tracing studies (Gu et al., 2002). NGN3 protein is detected largely during the 2nd trimester and is not seen in mature islet cells. $Ngn3^{-/-}$ mice show no islet development (Gradwohl et al., 2000) and transgenic overexpression of ngn3 results in the activation of an islet differentiation program in vivo as well as in cultured pancreatic duct cell lines (Herrera et al., 2002; Huang et al., 2000; Noguchi et al., 2006). The number of $NGN3^{+}$ cells increases and peaks at e 15.5, after which, expression gradually declines but is still detectable in the neonatal period (Gasa et al., 2004; Heremans et al., 2002; Schwitzgebel et al., 2000). Ngn3 expression or immunopositivity is not seen in insulin- and glucagon-producing cells, suggesting that ngn3 expression is not necessary for post-natal islet function (Gasa et al., 2004; Heremans et al., 2002; Schwitzgebel et al., 2000).

Although expression of neurogenin3 in the adult mouse pancreas has not been reported as yet, it has been suggested that islet regeneration in adult organisms recapitulates embryonic developmental pathways (Bonner-Weir et al., 1993).

Recently, it was reported that NGN3 immunopositivity is not detected during pancreas regeneration (Lee et al., 2006). This study also demonstrates that even after administration of the β-cell trophic glucagon-like peptide-1 receptor agonist exendin-4, NGN3 immunopositivity was not seen (Lee et al., 2006). These investigators, however, did not look at ngn3 transcripts during regeneration (personal communications with Doris A. Stoffers). We observed that ngn3 transcript is detectable during development, post-natal life as well as during pancreatic regeneration following partial pancreatectomy. However, no immunopositive cells were visualized during regeneration, consistent with previous report using ngn3-EGFP mice (Lee et al., 2006). We reasoned that ngn3 transcripts may be post-transcriptionally regulated and looked at expression of small RNA molecules (microRNAs) that have recently been identified as important regulators of post-transcriptional gene expression (Carthew, 2006; Engels and Hutvagner, 2006). MicroRNAs (miRNAs) are approximately 22-nucleotides long, evolutionary conserved class of non-protein-coding RNA molecules. These are known to act by negatively regulating gene expression at the post-transcriptional level (Alvarez-Garcia and Miska, 2005; Lagos-Quintana et al., 2002; Lau and Lai, 2005) either by blocking translation through incomplete binding to the 3′UTR of their target mRNA, as in *C. elegans*, or, by directing degradation of the target mRNA, as in *Arabidopsis thaliana*. It is believed that this decision between translational repression or target mRNA degradation is taken based on the level of complementarity between miRNA seed-sequence (first 2 to 8 bases of miRNA) and binding site on target mRNA. Presently, several hundreds of such miRNAs have been identified in the mouse genome and fewer of these have been validated (Berezikov et al., 2006). Studies carried out in the last few years indicate importance of miRNAs in regulation of insulin secretion (Poy et al., 2004), adipocyte differentiation (Esau et al., 2004) and neural stem cell fate (Smirnova et al., 2005). We now are beginning to understand that miRNAs play an important role in gene regulation and protein expression, a process that is delicately orchestrated during embryonic development. We carried out miRNA profiling of developing and regenerating pancreas to gain insights into mechanisms that regulate islet β-cell regeneration. High expression of miRNAs targeting ngn3 (miR-15a, miR-15b, miR-16 and miR-195) during pancreas regeneration indicates a possible mechanism of post-transcriptional regulation of ngn3.

Materials and methods

Mice breeding and isolation of developing pancreas

Six- to 8-week-old FVB/NJ mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained at the experimental animal facility of National Center for Cell Science according to guidelines outlined by the Institute's animal care and use committee. Breeding pairs were set and pregnancy was confirmed by observing vaginal smears. Pregnant females and newborn mice were euthanized at pre-defined intervals and pancreatic buds or pancreas was carefully dissected out using a stereo microscope. Pancreatic tissue samples at each of these time points were taken for RNA isolation and immunostaining. For RNA isolations, pancreatic samples were collected in Trizol (Invitrogen, Carlsbad, CA). Isolated islets or tissues for immunostaining were fixed in 4% freshly prepared paraformaldehyde and taken for immunocytochemistry.

Pancreatectomy and isolation of regenerating pancreas

Pancreatectomy (Px) was performed on 6- to 8-week-old male FVB/NJ mice following the procedures described elsewhere (Hardikar et al., 1999). Briefly, ketamine (150 mg/kg) and xylazine (10 mg/kg) were given intraperitoneally to anesthetize the mice. The abdomen was opened through a left lateral incision (as shown in Fig. S1) and around 70% of the total pancreatic tissue (as confirmed in a pilot study) was carefully removed. Incisions were closed using 4-0 absorbable sutures (Davis-Geck, Manati, PR) and autoclip wound clipper (BD, Franklin Lakes, NJ). Topical ointment (Soframycin®, Aventis Pharma. Ltd., Pune, India) was applied over the sutured wounds following surgery and animals were administered analgesics (Buprenorphine 0.05 mg/kg every 12 h for 3 days). To estimate the number of proliferative nuclei, 3 mice at each time point were injected with 200 mg/kg of BrdU, 6 h prior to euthanasia. Animals were sacrificed at predefined time points and regenerating pancreas were removed for RNA isolation or fixed in freshly prepared 4% paraformaldehyde. BrdU incorporation was detected every day using a monoclonal antibody (Sigma, St. Louis, MO) during first 10 post-operative days.

RNA isolation and quantitative real-time PCR

Tissue samples were homogenized and frozen in Trizol (Invitrogen, Carlsbad, CA). RNA was isolated as per the manufacturers' instructions, measured on ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and taken for reverse transcription/quantitative real-time PCR. First strand cDNA synthesis was carried out using 'high capacity cDNA archive kit' (Applied Biosystems, Foster City, CA). PCR was performed in 5 μl or 10 μl total volume in 96-well plates using cDNA prepared from 100 ng of total RNA on a