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MicroRNA Profiling of Developing and Regenerating Pancreas Reveal Post-transcriptional Regulation of Neurogenin3

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Abstract

The mammalian pancreas is known to show a remarkable degree of regenerative ability. Several studies till 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 show 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 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 Neurogenin-3 (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 over-expression 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 neurogenin-3 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, miR16 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 were 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 6-8 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 supplementary 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 hours 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 hours 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 7500 FAST real time PCR cycler (Applied Biosystems, Foster City, CA). Primers and probes were Assay-on-Demand (Applied Biosystems, Foster City, CA). For estimation of fold-changes by qRT-PCR when the initial transcript levels were undetectable, the initial Ct value was assigned to be 38, which would lead to a possible underestimation of the actual fold-change. All qRT-PCR results were normalized to 18S (VIC-labeled) ribosomal RNA carried out in duplex reaction (with FAM labeled target gene probes) to correct for any differences in RNA input. Conventional pcr was carried out on reverse transcribed samples using AmpliTaq Gold (Applied Biosystems, Foster City, CA) and known / published primers (Primer sequences for β-actin and NeuroD are available from the authors upon request). All pcr reactions were analyzed after 35 cycles of amplification.

For microRNA detection, complete mouse miRNA panel (Applied Biosystems, Foster city, CA) including 283 miRNAs was used. Reverse transcription was carried out using mature miRNA-specific primer sets (Applied Biosystems, Foster City, CA) and microRNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on Applied Biosystems 7500 FAST system using miRNA-specific taqman-based probe-primer sets (Applied Biosystems, Foster City, CA). All sample plates included positive, negative and endogenous controls supplied by the manufacturer in duplicate.

Immunostaining and confocal microscopy

Mouse anti-insulin antibody (Linco Research Inc, MO) and mouse anti-glucagon (Sigma, St. Louis, MO) antibodies were used at 1:100 dilutions. Rabbit anti-NeuroD (Chemicon Int. Inc, Temecula, CA) and mouse anti-Neurogenin3 antibody (BD Biosciences, San Diego, CA) were used at 1:100 dilution. Alexa-Fluor 488, Alexa-Fluor 546 and Alexa-Fluor 633 $F(ab')_2$ secondary antibodies (Molecular Probes, OR) were used at 1:200 dilution. Hoechst 33342 was used to visualize nuclei. Cells were fixed in 4% fresh paraformaldehyde, permeabilized with chilled 50% methanol, blocked with 4% normal donkey serum and then incubated with antisera. Primary antibodies were incubated overnight at 4°C, washed with PBS and then incubated with the secondary antibodies at 37°C for 1 hour. Slides were washed extensively in PBS and mounted in Mowiol. Confocal images were captured using a Zeiss LSM 510 laser scanning microscope using a 63X/1.3 oil objective with optical slices ~0.8 μm. Magnification, laser and detector gains were set below saturation and were identical across samples. Results presented are representative fields confirmed from at least 5 different experiments.

Cell culture and anti-sense studies:

Since levels of miRNAs specific for ngn3 increased by day 3 after regeneration, we took out regenerating pancreas 24 hours after pancreatectomy. The regenerating pancreatic tissue obtained was finely chopped and digested with collagenase to prepare single cell suspension. These were transfected with antisense miRNAs (Ambion, Austin, TX) specifically designed and validated against miR-15a, miR-15b, miR-16 and miR-195 or mutants in which the 'seed sequence' (first 8 bases) were altered. The seed sequence for miR-15a, -15b, -16 and -195 is: UAGCAGCAC while the seed sequence designed for mutant miR was ACUGCAGUG. siPORT *NeoFX*, a lipid based reagent (Ambion, Austin, TX) was used for transfection as per the manufacturers recommendations. Briefy, siPORT *NeoFX* was diluted in Opti-MEM 1 medium and incubated at room temperature for 10 min. MicroRNA inhibitors or mutant-anti-miRNA were diluted in Opti-MEM to a final concentration of 30 nM. Diluted RNA and diluted siPORT *NeoFX* were mixed by gentle pipetting and incubated at room temperature for 10 min. The RNA/siPORT *NeoFX* complexes were then distributed to each well and overlaid with cell suspension. Cells harvested after 4 or 6 days of transfection were taken for transcript analysis (neuroD and nkx2.2) and immunostaining.

Target prediction and cluster analysis

Since mammalian miRNAs are generally thought to recognize 3'UTR of target mRNA via partial complementarity, we used carefully designed computational approaches to predict mRNA targets for mammalian miRNAs. Two target search engines from Memorial Sloan-Kettering Cancer Center (http://www.microrna.org/), miRanda software and target analysis by PicTar (http://pictar.bio.nyu.edu/) were mainly used to confirm

targets for specific miRNAs. Normalized data sets from realtime pcr analysis of miRNA expression profiles were taken as input data for bi-directional clustering. Bi-directional clustering is one of the most widely used algorithms to recognize patterns in datasets with similar expression profiles. Since functional modules of genes are generally regulated together, such modules can be identified from the similarity of their expression patterns in a bi-directional analysis. Two-way clustering was performed in MatLab^{TM}, using the Bioinformatics Tool-box (MatLab^{TM} v 7.0, R 14), which basically groups the samples with similar matching gene profiles together across the X-axis. Genes within these grouped samples that show similar expression patterns are grouped together along the Y-axis. Bi-directional clustering thus offers an important tool to assess closely related samples as well as similar gene expression pattern within these sample groups.

Results and Discussion

Islet hormones show similar gene expression patterns during development and regeneration

Pancreatic regeneration after pancreatectomy has been well characterized (Bonner-Weir et al., 1993; Bonner-Weir and Weir, 2005; Hardikar, 2004; Hardikar et al., 1999) using several transgenic and knockout mice. We assessed islet (pro-) hormones and transcription factors expressed during mouse pancreatic development by multiplex quantitative real-time pcr (qRT-PCR). We found that mouse pro-insulin2 and pro-glucagon gene transcripts are expressed at detectable levels as early as e 10.5 (Fig. 1A). By e 16.5, we detect a massive (>1000-fold) increase in the levels of gene transcripts for these 2 islet hormones (Fig. 1A). This is rather expected as most of the islet

development/differentiation occurs during the second trimester (Sanders and Rutter, 1974; Watada, 2004). There is no significant change later on in abundance of pro-insulin2 and pro-glucagon transcripts during post-natal period or by 8 weeks (Fig. 1A). We then carried out pancreatectomy on 8-week-old mice (Supplementary Fig. S1) and removed ~70% of total pancreatic mass. This resulted in around 10- to 100-fold decrease in levels of pancreatic islet hormone gene transcripts (Fig. 1A) by 3 days post-Px. Pro-insulin2 gene transcripts increased and stabilized to pre-operative levels by end of 4 weeks. Pancreatic islet regeneration-associated gene transcripts (reg3a and reg3g) showed 100-fold more abundance (Fig. 1A) at 3 days post-Px. By 4 weeks after pancreatectomy, levels of reg3a and reg3g stabilized down to pre-operative levels. Since pancreatic regeneration is known to involve islet-, duct- or acinar-cell proliferation, we looked at BrdU-incorporation in these cells during pancreas regeneration. Following partial pancreatectomy, we observed increase in pancreatic duct as well as islet cell proliferation (Fig. 1B-E). BrdU-labeled nuclei were first seen in small and large ducts and then later on in islets (Fig. 1E). Recent promoter-based lineage tracing studies have demonstrated that reconstitution of pancreatic mass following surgical resection occurs primarily via proliferation of endocrine, exocrine and ductal tissue (Brennand et al., 2007; Desai et al., 2007; Strobel et al., 2007), which give rise to respective cells in the regenerated pancreas. We observed BrdU immunopositivity in each of these pancreatic ‘compartments’ and believe that proliferation of exocrine, endocrine as well as ductal-cells plays an important role in reconstitution of the entire organ.

Pancreatic transcription factors upstream of ngn3 show similar expression during development and regeneration

We looked at expression of pancreatic islet hormones in day 1 neonates (Fig. 2A) as well as 26-days post-Px in adult mice (Fig. 2B). There are no differences in the % of insulin- and glucagon-immunopositive cells between islets isolated from day-1 neonates (67 $\pm$ 2%) or day-26 regenerated pancreas (76 $\pm$ 3%; $p>0.05$). These data demonstrate that *de novo* development of hormone-producing cells or pancreatic regeneration following pancreatectomy leads to generation of islets that contain similar number of hormone-producing cells. To further understand similarities in endocrine pancreas development and regeneration, we assessed the transcript levels of several transcription factors expressed during pancreas development (illustrated in Fig. 2C). We found that pancreatic transcription factors such as hnf3β, pdx1 as well as ngn3 (which marks endocrine progenitor cells), show similar increase in transcript abundance during regeneration (Fig. 2D-F). Hnf3β, pdx1 and ngn3 gene transcripts that are expressed at relatively lower abundance by e 10.5 are seen to increase by e 16.5. The level of gene transcripts for these transcription factors in 8 week old adult pancreas is similar to that seen in e 10.5 pancreatic buds. In adult mice, gene transcripts for pdx1 and ngn3 show a similar transient increase immediately after pancreatectomy (Fig. 2E, F). Surprisingly, when we looked at pancreatic transcription factors downstream of ngn3, we did not see a similar trend for neuroD (Fig. 3A) or nkx2.2 (Fig. 2G). NeuroD immunopositivity was detectable during development at e 15.5 – e 17.5, but not during regeneration (Fig 3B).

Differences in protein expression are actually due to post-transcriptional regulation of ngn3 gene transcripts

Neurogenin3 is a bHLH transcription factor that has been demonstrated to be essential for normal development of islet cells. Previous studies have demonstrated that NGN3 immunopositive cells, which are evident during pancreas development, are not seen during pancreas regeneration (Lee et al., 2006). In this study as well, we detect NGN3 immunopositive cells during development but not at any point during pancreas regeneration (Fig. 4A). Since the half-life of NGN3 protein may be very short, Lee et al also looked at ngn3 expression after regeneration using ngn3-EGFP transgenic mice (Lee et al., 2006). They could not detect any GFP-positive cells following pancreatectomy and therefore concluded that neurogenin3 is not activated during pancreas regeneration. Interestingly, when we looked at the construct used to derive the ngn3-EGFP mice (Lee et al., 2002) we noticed that these mice were generated by inserting the EGFP tag in coding region of ngn3 gene, while maintaining the 5'- as well as 3'-UTR intact. Since we detected ngn3 transcript at very high abundance (Cycle threshold (Ct) value 19-21) during regeneration we thought that ngn3 must be post-transcriptionally regulated via microRNAs that could bind to 3'UTR of endogenous ngn3 (or ngn3-EGFP) mRNA. We therefore decided to look at the expression of 283 known / validated miRNAs during development and regeneration. Two-dimensional cluster analysis of miRNAs targeting transcription factors (outlined in figure 2C) indicated that miRNA profiles of regenerated pancreas (day 26 and day 16 post-Px) were similar to adult 8 week old mice. Such a clustering pattern indicates that miRNA gene expression patterns between day 26 (and 16) post-px and adult mice have similar expression profiles. Likewise, day 9 post-Px

miRNA profiles were very similar to that of e 16.5 developing pancreas, but not similar to those of the 3 day post-Px mice or adult mice pancreas (Fig. 4B). These data demonstrate that similarities in development and regeneration exist even at the 'microRNA level'.

We then looked at miRNAs that could specifically bind to ngn3. We came up with 7 microRNAs that could potentially bind to 3'UTR of ngn3. Of these, 4 miRNAs: miR-15a, miR-15b, miR-16 and miR-195 did not show any significant change during development or on day 1 (Fig. 4C). However, after partial pancreatectomy these 4 miRNAs were seen to be expressed at least 200-fold more at day 3 post-Px as compared to those observed during development (Fig. 4C). All of these miRNAs that bind to ngn3 carry a specific seed sequence: UAGCAGCA, which refers first 2 to 8 bases of these microRNAs. We also looked at miRNAs that showed no similarity with this seed sequence. Four such miRNAs, some of which target other pancreatic transcription factors but show no similarity to ngn3-specific miRNAs, were not upregulated at this time (Fig. S2B). Anti-miRs, which are anti-sense miRNAs to 22 nt sequences of miR-15a, -15b, -16 and -195 were obtained from Ambion. We also generated mutant anti-miRs, which have a distinctly different seed sequence and carry < 25% similarities in remaining 12-14 bases after the seed sequence. We transfected miRNA-specific anti-sense RNAs into cells isolated from regenerating pancreas (day 1 post-Px) and looked at expression of downstream transcription factors such as nkx2.2 and neuroD. We could detect expression of neuroD (Fig 4D) as well as nkx2.2 (not shown) following transfection of anti-sense miRNAs into day-1 regenerating pancreatic cells.

Untransfected cells maintained under similar culture conditions (Fig. 4D) or mutant anti-miRs transfected into day 1 regenerating pancreatic cells did not show expression of neuroD. NGN3 immunopositivity was detected in 9.2 $\pm$ 1.3% of regenerating pancreatic cells that were transfected with specific miRNA inhibitors only but not in cells that were transfected with mutant anti-miRNA or untransfected cells. Nonetheless, it appears that expression of NGN3 in these cells resulted in appearance of downstream transcription factors such as neuro D (Fig 4D).

Our data indicates that transient increases in miRNAs that bind to ngn3 may be involved in inhibiting ngn3 translation during regeneration. Absence of NGN3 protein during regeneration also results in diminished expression of its downstream gene targets. Most significant differences were seen in the expression of neuroD and nkx2.2. Nkx2.2 was not detectable at 3-, 9- or 16-days post-Px (Fig. 2G). We also could not detect expression of neuroD (Fig. 3A) during regeneration. However, other mature islet transcription factors such as isl1, nkx6.1and pax6 are detected at lower levels (Ct values up to mid-30s) during regeneration. These studies indicate that though NGN3 protein is not produced during regeneration (Fig 4A), there are no differences in expression of mature islet hormones (Fig 1A and Fig 2A,B) or transcription factors (Fig. 2). We demonstrate that inhibition of ngn3-translation affects transcription of downstream genes such as neuroD (Fig 3A) and nkx2.2 (Fig 2G). However, when cells isolated from regenerating pancreas were transfected with anti-miRNAs to miR-15a, miR-15b, miR-16 and miR-195, we see expression of NGN3 and transcription of downstream genes such as neuroD (Fig 4D). Mutant inhibitors did not result in expression of neuroD (Fig 4D). Since these

miRNAs inhibit ngn3 translation, we decided to look at the role of these miRNAs during pancreas development. We overexpressed ngn3-specific miRNA duplexes in e12.5 pancreatic buds and looked at expression of islet endocrine cells at 4-days in vitro. We observed that overexpression of miRNAs in developing pancreatic buds led to reduction in the number of insulin and glucagon-producing cells in developing pancreas (Fig S3). Developing pancreatic buds overexpressing these miRNAs showed significantly lower number of insulin and glucagon-producing cells (5.2 $\pm$ 1%) as compared to control pancreatic buds (15.1 $\pm$ 1.8%, $p<0.001$). Our data demonstrate that differences in gene expression during development and regeneration exist at the level of post-transcriptional regulation of neurogenin3, a gene that marks pro-endocrine cells during pancreas development.

What may be the reasons for such differences during development and regeneration? One plausible explanation may be that pancreatic regeneration in mice following pancreatectomy does not occur from the pancreatic "stem cell" as is known to happen during embryonic development. Some of the recent evidences indicate that replication of existing β-cells may be one such mechanism for β-cell renewal following pancreatectomy in adult mice (Dor et al., 2004). It is possible that a more "committed" population of pancreatic islet progenitor cells may be involved in generation of new hormone-producing cells by an alternate route that does not involve sequential activation of transcription factors. During embryonic development, lineage tracing as well as knockout studies presented by several groups have demonstrated that differentiation of a pancreas-specific "stem cell" into hormone-producing cell clusters involves temporal

expression of several pancreatic transcription factors. We believe that though such a temporal expression of transcription factors is essential for normal embryonic development, it may not be necessary during adult life. The pancreas seems to suppress islet neogenesis via stem cell pathway in the regenerating pancreas, following pancreatectomy. Analysis of expression of these 4 miRNAs in other tissues (heart, lungs, kidney and brain) does not change during this time (Fig. S2C). The pancreas-specific increases in miRNAs targeting ngn3 seem to prevent islet neogenesis via stem cells. We think that there exists at least one (if not many) alternative pathway(s) that contributes to islet neogenesis following pancreatectomy in mice. Two recent reports using genetic lineage tracing in mice provide evidence that following partial pancreatectomy, pre-existing acinar cells contribute to generation of new acinar (but not islet) cell types (Desai et al., 2007) and pre-existing islet β-cells contribute to generation of islet β-cells, as demonstrated earlier (Dor et al., 2004), but not acinar cells (Strobel et al., 2007). Thus islet neogenesis following pancreatectomy does not seem to occur via the major (acinar) pancreatic component. One begins to wonder about other pathways that may be involved in generation of new islets, following pancreatectomy in mice. Recent studies using serial thymidine analog labeling (Teta et al., 2007) or promoter-based gene expression studies (Brennand et al., 2007) provide evidence that all pre-existing insulin-expressing (β-) cells have similar potential to contribute to "new islets" during pancreas regeneration. Though these lineage tracing studies do not support (nor fully refute) the possible contribution of pancreatic duct cells, what seems evident is that the pancreatic islet β-cells themselves may be one of the major contributors to islet neogenesis following pancreatectomy. It appears to us that in presence of such a

"committed" progenitor cell (the β-cell itself), islet neogenesis from stem cells would be a less "favored" alternative. We demonstrate that active proliferation of duct-, islet- as well as exocrine-cells occurs following pancreatectomy in these mice (Fig 1B-E). Our data are in agreement with recent reports demonstrating proliferation of these pancreatic compartments (ductal, endocrine and exocrine) in restoration of the pancreas (Brennand et al., 2007; Strobel et al., 2007; Teta et al., 2007) and we do believe that in presence of this alternate pathway (β-cell to β-cells), an islet "stem-cell" dependent pathway is inhibited by tissue-specific increase in miRNAs targeting ngn3. We demonstrate here that microRNA mediated regulation of neurogenin3 expression allows regeneration of endocrine pancreas via an alternate pathway that may not involve neogenesis from pancreatic "stem cells".

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Legends.

Fig. 1. Pancreas development and regeneration. (A) Pancreatic islet hormones (pro-insulin2 and pro-glucagon) and regeneration genes (reg3a and reg3g) expression during development and regeneration. Data are mean ± s.e.m for 3 mice each and represent fold increase over detectable (Ct value of 38), as assessed by quantitative real time pcr. Eight-week old adult mice show no significant BrdU incorporation at day 0 (B) but increased proliferation in ducts (arrowheads) by day 3 (C). Regenerating pancreatic islets (arrows) show increased BrdU incorporation by day 10 (D). (E) $BrdU^+$ cells in small ducts (<50 μm internal diameter), large ducts (>50 μm internal diameter) and islets at different days following pancreatectomy are quantified and presented as mean ± s.e.m from at least 2 mice and 10 sections each. Bar represents 50 μm.

Fig. 2. Pancreatic transcription factors expressed during development and regeneration. Confocal optical section of pancreatic islets isolated from day 1 neonates (A) or day 26 post-pancreatectomy (B). Bar represents 50 μm. (C) Brief overview of temporal sequence of pancreatic transcription factors known to be expressed during development. Transcript abundance of pancreatic transcription factors as estimated by duplex quantitative real time pcr is plotted (D-I) as fold increase of mean ± s.e.m over detectable (Ct of 38).

Fig. 3. NeuroD expression during development and regeneration. (A) NeuroD transcript is detectable during development but not at any point during regeneration. Relative quantization presented here is a representative of 3 different biological

replicates. (B) During development a few NeuroD (red arrowheads) immunopositive cells show co-expression of islet hormones (insulin in green and glucagon in pink). However, we could not detect NeuroD immunopositive cells during regeneration though islet hormone-positive cells appeared (mostly closer to ducts) in the regenerating pancreas. Bar represents 20μm.

Fig. 4. Neurogenin3 expression during regeneration may be regulated by microRNAs. (A) Neurogenin3 protein is detectable in developing pancreatic buds at e 12.5, but not at day 3 (or other time points not shown) following pancreatectomy in mouse. (B) Heatmap for microRNA profiling of miRNAs that can potentially bind to different transcription factors (as outlined in Fig. 2C). Legend indicates the normalized Cycle threshold (Ct) value from black color, representing high expression (low Ct value) to white color, representing low expression (high Ct value) of the miRNA assessed. Intermediate expression is plotted in scales of red/orange. Cycle threshold of 40 is considered undetectable. (C) Four different miRNAs that can potentially bind to neurogenin3 are expressed at levels similar to adult in e 10.5 embryos, but are rapidly over expressed at 3 days after pancreatectomy. (D) NeuroD transcript is not detected in freshly isolated (day-1 post-Px) cells or after 4 days in vitro without or with transfection of mutant anti-miRNA. However, after co-transfection with inhibitors for miRNAs that target ngn3, these cells show expression of neuroD. Data represents analysis from 4 biological replicates. Bar is 10μm.

Fig. S1. Partial Pancreatectomy in FVB/NJ mice. A schematic showing actual procedure in which 70% of total pancreatic mass is removed. Details of the procedure are presented under Materials and Methods section of this manuscript.

Fig. S2. MicroRNAs targeting ngn3 show tissue-specific expression. A) As shown in fig 4C, four different miRNAs that can potentially bind to neurogenin3 are expressed at levels similar to adult in e10.5 embryos, but are rapidly over expressed at 3 days after pancreatectomy. **B)** At the same time, we assessed expression of 4 other miRNAs that do not bind to ngn3. All of these 4 miRNAs carry seed sequence with no similarity to the seed sequence of ngn3-targeting miRNAs. We found that miRNAs, which do not target ngn3, are not upregulated during the same time course. **C)** Since miRNAs targeting ngn3 were seen with increased abundance in pancreatectomized mice, we look at the tissue specificity of these miRNAs. We found that ngn3-specific miRNAs showed increased expression in the pancreas (fig 4C or fig S2A), but not in other tissues such as brain, heart lungs or kidneys. Taken together, these data suggest that ngn3-targeting miRNAs are specifically seen with increased abundance in regenerating pancreas.

Fig. S3. Overexpression of miRNAs targeting ngn3 leads to reduction in number of hormone-producing cells during development. To assess a role of ngn3-targeting miRNAs during pancreatic development, we isolated cells from embryonic day (e) 12.5 pancreatic buds and allowed them to grow in vitro without or after overexpression of miRNA duplexes for miR-15a, -15b, -16 and -195. Insulin- and glucagon-producing

cells were counted after 4 days and are represented here as % of total cells. Data represents scans from 3 litters.