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Proinflammatory cytokines induce endocrine differentiation in pancreatic ductal cells via STAT3-dependent NGN3 activation

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SUMMARY

A major goal of diabetes research is to develop strategies that replenish pancreatic insulin-producing beta cells. One emerging strategy is to harness pancreatic plasticity—the ability of pancreatic cells to undergo cellular interconversions—a phenomenon implicated in physiological stress and pancreatic injury. Here we investigated the effects of inflammatory cytokine stress on the differentiation potential of ductal cells in a human cell line, in mouse ductal cells by pancreatic intraductal injection, and during the progression of autoimmune diabetes in the non-obese diabetic (NOD) mouse model. We find that inflammatory cytokine insults stimulate epithelial-to-mesenchymal transition (EMT) as well as the endocrine program in human pancreatic ductal cells

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AUTHOR CONTRIBUTIONS

Conceptualization, I.A.V., E.D. and A.K.K.T.; Methodology, I.A.V., E.D. and A.K.K.T..; Investigation, I.A.V. and E.D., M.K.G., J.S., A.K.K.T.; Writing – Original Draft, I.A.V.; Writing – Review & Editing, I.A.V., E.D., M.K.G., J.S., A.K.K.T and R.N.K.; Funding Acquisition, R.N.K.; Resources, Conceptual discussions; R.N.K.; Supervision, A.K.K.T. and R.N.K.

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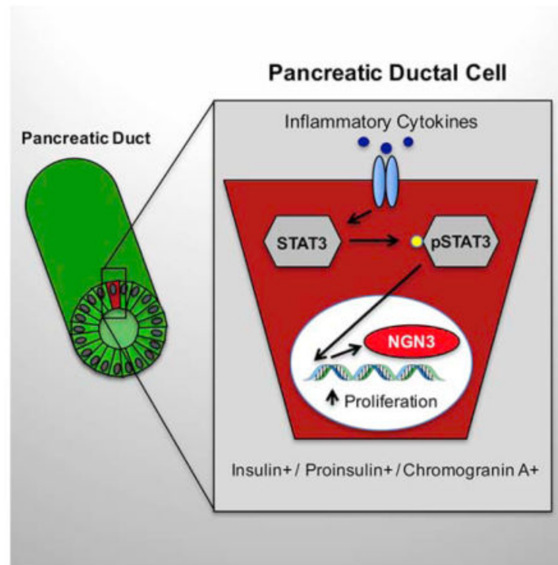
via STAT3-dependent NGN3 activation. Furthermore, we show that inflammatory cytokines activate ductal-to-endocrine cell reprogramming in vivo independently of hyperglycemic stress. Together, our findings provide evidence that inflammatory cytokines direct ductal-to-endocrine cell differentiation, with implications for beta cell regeneration.

Graphical Abstract



INTRODUCTION

The ability of the pancreas to host an array of intrapancreatic cellular conversions offers promise to the fields of regenerative biology and diabetes research. A growing and compelling line of evidence in rodents and humans suggests that pancreatic cell plasticity allows the generation of insulin-producing cells from non-beta cell sources, particularly during times of need (e.g. beta cell loss, pancreatic injury or metabolic stress) (Valdez et al., 2015). Various groups have argued for or against this phenomenon in an ongoing debate about the origin of the newly formed beta cells (Dor et al., 2004; Kopp et al., 2011; Kulkarni et al., 2004; Nir et al., 2007; Solar et al., 2009; Xiao et al., 2013). Given its potential therapeutic applications, it is timely to address these questions to obtain a better understanding of the mechanisms that control pancreatic cell plasticity.

The pancreas is a highly diversified glandular organ that houses two major cell types which contribute to the endocrine and exocrine compartments. The former constitutes ~2% of the pancreas and consists of five hormone-secreting cells—alpha, beta, delta, gamma (PP), and epsilon cells—that make up the islets of Langerhans. The remainder of the pancreas is made up of the exocrine portion and contains acinar and ductal cells, which are responsible for digestive enzyme and bicarbonate secretions. Pancreatic cell plasticity has been reported in most of these cells, both endocrine and exocrine, using a variety of models and experimental approaches (Valdez et al., 2015). Furthermore, numerous studies have reported that pancreatic cell interconversions are mediated via the emergence of NGN3, a key endocrine

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progenitor transcription factor necessary for endocrine cell specification (Rukstalis and Habener, 2009).

While there are some links between inflammatory stress and exocrine ductal cell transdifferentiation, detailed cell and molecular mechanisms have not been fully established. Hence, we sought to investigate whether stress, induced specifically by proinflammatory cytokines, TNFα, IL-1β, and IFNγ—critical cytokines implicated in the pathogenesis of both type 1 and type 2 diabetes—have the ability to direct the differentiation of human and mouse pancreatic ductal cells towards the endocrine lineage. Here, we report that inflammatory signaling induces epithelial-to-mesenchymal transition (EMT) and the upregulation of the endocrine progenitor marker, NGN3, via STAT3 activation in the human ductal epithelial cell line PANC-1. By performing a parallel in vivo approach, a pancreatic intraductal injection of the same cocktail of proinflammatory cytokines in C57BL/6 mice, we show that the acute inflammatory cytokine insult on pancreatic ductal cells is sufficient to stimulate ductal-to-endocrine cell reprogramming. Finally, by following the progression of autoimmune diabetes in the non-obese diabetic (NOD) mouse model, we demonstrate that ductal cell proliferation, as well as the emergence of NGN3 and phosphorylated STAT3 (pSTAT3) expression in pancreatic ductal cells, correlates with the presence of serum cytokine levels and pancreatic immune cell infiltration, independent of hyperglycemic stress.

RESULTS

Inflammatory cytokines induce epithelial-to-mesenchymal transition in human ductal cells

The human ductal epithelial cell line, PANC-1, a commonly used cell line for in vitro differentiation studies (Hardikar et al., 2003; Lefebvre et al., 2010; Wu et al., 2010), was treated either with a single cytokine or different combinations of the three proinflammatory cytokines, TNFα, IL-1β, and IFNγ for 24, 48 or 72 hrs. The optimal dose of cytokines (Cx) used for stimulations was finalized following dose optimization studies and is referred to as [1X] (TNFα [50ng/mL]; IL-1β [25ng/mL]; IFNγ [100ng/mL]) (Figures S1A–C).

To assess cytokine-induced stress, mRNA levels of *iNOS* and *CHOP* were measured by qPCR in untreated or [1X] cytokine-treated PANC-1 cells. These analyses demonstrated an increase in *iNOS* and *CHOP* gene expression at all time points (Figure 1A). Furthermore, immunofluorescence analyses revealed a significant increase in the apoptosis marker, cleaved caspase-3, following cytokine stimulations relative to untreated controls (Figure 1B; left, upper and lower panels).

An important decision progenitor cells must make is to proliferate or differentiate, as many cells, including beta cell progenitors (Kim et al., 2015), must temporarily defer their proliferative abilities while undergoing differentiation (Gingold et al., 2014; Zhu and Skoultchi, 2001). To assess proliferation, we performed Ki67 immunostaining analyses (Figure 1B; right, upper and lower panels) and assessed cell growth (Figure S1D). Cytokine-treatment of PANC-1 cells led to a significant decrease in the proliferation marker, Ki67, at 72hrs (Figure 1B; right, upper and lower panels) and reduced cell numbers at all time-points (Figure S1D) relative to untreated controls. In addition, observation by brightfield microscopy revealed a morphological change in cytokine-treated cells. Untreated PANC-1

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cells exhibited a normal epithelial-like morphology (Lieber et al., 1975), while cytokine-treated cells appeared more mesenchymal-like at all time-points (Figure 1C; representative data shown for 72hrs) (Attali et al., 2007).

Since epithelial-to-mesenchymal transition (EMT) is associated with the differentiation of epithelial cells toward a pancreatic endocrine progenitor state (Rukstalis and Habener, 2007), we assessed the levels of the ductal cell marker, carbonic anhydrase II (*CA-II*), the epithelial marker, e-cadherin (*CDH1*), and the mesenchymal marker, *SNAIL2* by qPCR. These analyses demonstrated a significant decrease in mRNA levels of ductal cell marker, *CA-II*, and epithelial marker, *CDH1*, at all time points, as well as a significant increase in mesenchymal marker, *SNAIL2* (Rukstalis and Habener, 2007) at 72hrs (Figures 1D–F). Protein immunoblot analyses also revealed a decrease in CDH1 and an increase in SNAIL2 in PANC-1 cells treated with cytokines at 72hrs relative to untreated controls (Figures 1G and 1H). Together, these data suggest that proinflammatory cytokine-induced stress triggers the loss of ductal cell identity in the human epithelial ductal cell line, PANC-1, and stimulates their differentiation from an epithelial cell type towards a mesenchymal-like cell.

Proinflammatory cytokines upregulate NGN3 in human ductal cells via STAT3 signaling

To further address the hypothesis that cytokine-induced stress induces the differentiation of the human ductal cell line, PANC-1, towards the endocrine cell lineage, we assessed the expression of the endocrine progenitor marker NGN3. Following [1X] cytokine stimulation, qPCR analyses revealed an upregulation of *NGN3* expression at all time points in cytokine-treated conditions relative to untreated controls (Figure 2A; dose optimizations S2A–C). These results were corroborated at the protein level by immunofluorescence (Figures 2B and 2C). In addition, gene expression of other endocrine markers, *NKX6.1* (Figure S2D–G) and *HNF6* (Figure S2H), were also upregulated in PANC-1 cells upon cytokine stimulation. To assess the specificity of cytokine-induced endocrine progenitor upregulation in human ductal cells, we used another cell line of similar (endodermal) developmental origin—the human liver cell line, HepG2. Stimulation with the same cocktail of proinflammatory cytokines did not significantly affect the mRNA expression levels of *NGN3*, *NKX6.1* or *HNF6* relative to untreated controls at any time-point (Figures S2I–K).

Next, to investigate the gene expression levels of *NGN3* in PANC-1 cells after cytokine removal, we divided the experiment into three groups: 1) 96hrs untreated control (Ctrl); 2) 48hrs without cytokines + 48hrs with cytokines, (Cx−|Cx+); and 3) 48hrs with cytokines + 48hrs without cytokines (Cx+|Cx−). As expected, there was a down-regulation in the expression of the stress marker, *iNOS* gene, after cytokine removal (Cx+|Cx−), but, interestingly, *NGN3* mRNA and protein levels, as well as *NKX6.1* and *HNF6* mRNA levels remained elevated even after cytokine removal (Figure 2D–E and S2L–M).

qPCR analyses of the three proinflammatory cytokines revealed an autocrine upregulation of *TNFα* and *IL-1β* gene expression as has been previously reported (Brewington et al., 2001) but not that of *IFNγ* (Figure 2F). These observations could, in part, explain the persistent and elevated expression of endocrine genes even upon cytokine removal (Figure 2D–E and S2L–M). To test this, we treated PANC-1 cells with a cocktail of specific receptor antagonists (TNFα R [25μM]; IL-1 R [50μM]; IFNγ R [25μM]) after cytokine removal and examined

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NGN3 protein expression by immunoblot analysis. We found that NGN3 expression is markedly decreased in the presence of cytokine receptor antagonists (Cx+|Ag) relative to the cytokine-treated (Cx−|Cx+) and cytokine-depleted (Cx+|Cx−) conditions (Figure 2E).

Since NGN3 is a downstream effector of STAT3 (Baeyens et al., 2006; Kaucher et al., 2012; Lemper et al., 2015), and STAT3 has been shown to be activated by inflammatory signaling (Ivashkiv and Donlin, 2014; Yu et al., 2009), we assessed whether NGN3 upregulation in our proinflammatory cytokine-treated PANC-1 cell model is mediated through STAT3 activation. To this end, we used a pSTAT3 small-molecule inhibitor (Stattic, Selleckchem) and assessed the levels of pSTAT3 by immunoblot analyses. Our results demonstrate that [1X] cytokine stimulation at 72hrs induces an increase in pSTAT3 relative to untreated and DMSO-treated controls, which is abrogated upon Stattic [5μM] and decreased to control levels upon Cx+Stattic [5μM] treatment (Figure 2G). Importantly, NGN3 protein expression is upregulated only upon [1X] cytokine stimulation relative to untreated and DMSO controls, but not when cells are treated with Stattic [5μM] or Cx+Stattic [5μM] (Figure 2G). Together, our results indicate that proinflammatory cytokines upregulate NGN3 expression in the human ductal cell line, PANC-1, and this requires the activation of pSTAT3.

Pancreatic intraductal injection of proinflammatory cytokines stimulates ductal cell proliferation and an increase in insulin$^+$ ductal cells in mice

To explore the effects of proinflammatory cytokine stimulation in vivo, we performed a single injection of the cocktail of cytokines (TNFα, IL-1β, IFNγ) directly into the pancreatic duct of 8–10 week old C57BL/6 mice. We performed these injections at a [1X], [2X], or [10X] dose, and compared them to saline-injected controls (Figures 3A and S3A).

Following the intraductal injections, we measured blood glucose levels weekly for 3 weeks and injected BrdU intraperitoneally in all mice at 3 days and at 5 hours prior to sacrifice (Figures 3B and S3A). The lack of significant differences in blood glucose levels among the groups ruled out possible effects induced by hyperglycemic stress (Figure 3B). Three weeks post-injection, we harvested the pancreata and performed immunohistochemistry.

Immunofluorescence analyses demonstrated increased levels of ductal cell proliferation, as measured by co-immunostaining for BrdU and the ductal marker (DBA) at [2X] and [10X] concentrations relative to saline-injected mice (Figures 3C and 3D). Strikingly, immunostaining analyses also revealed an increase in the number of insulin$^+$ ductal cells in a dose-dependent manner in cytokine-injected mice (Figures 3E–H and S3B–C). Moreover, to investigate whether these insulin$^+$ ductal cells displayed characteristics of true beta cells, we co-immunostained insulin with other markers of beta cell identity—secretory granule protein, Chromogranin A, (Lukinius et al., 2003), and the insulin precursor, proinsulin (de la Rosa and de Pablo, 2011). Our results revealed that the number of Chromogranin A/DBA (Figure 3E and 3F) and Proinsulin/DBA (Figure 3G and 3H) double+ cells significantly increased in all cytokine injection groups relative to saline-injected controls. In addition, we found that the majority of insulin$^+$ ductal cells also expressed proinsulin (Figure 3G and 3H), whereas the number of Chromogranin A$^+$ ductal cells was higher than that of insulin$^+$ ductal cells in all groups (Figure 3E and 3F). Since Chromogranin A is also expressed in the secretory granules of other endocrine cells (Lukinius et al., 2003), our results suggest that

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some ductal cells may adopt a non-beta endocrine cell fate. In addition, we did not observe NGN3$^+$ ductal cells in these sections (data not shown), in contrast to our findings in vitro. These findings suggest that the acute effects of a single proinflammatory cytokine injection is unlikely to be sufficient to maintain endocrine cells in an undifferentiated state for a prolonged period, perhaps due to a transient expression of NGN3, in pancreatic ducts.

Ductal cell proliferation and emergence of NGN3$^+$ and pSTAT$^+$ ductal cells correlate with immune cell infiltration, and not hyperglycemia, in NOD mice

Previous studies using the non-obese diabetic (NOD) mouse model have reported an increase in pancreatic ductal cell proliferation and in the number of insulin$^+$ and glucagon$^+$ ductal cells in diabetic conditions (O'Reilly et al., 1997). Furthermore, it has been reported that cells harvested from the ductal epithelium of NOD mice contain progenitor cells capable of clustering and forming functional islets in vitro that can be re-implanted into NOD mice to restore normoglycemia (Ramiya et al., 2000).

Based on these reports, we sought to investigate whether chronic pancreatic immune cell infiltration can generate NGN3$^+$ endocrine progenitor cells in the pancreatic ducts of NOD mice at various stages of autoimmune diabetes progression. We harvested the pancreata of mice at three pre-diabetic (3, 8 and 12 weeks of age) and two diabetic stages (new onset diabetes at 1–2 weeks post-diabetes onset and established diabetes at 5–6 weeks post-diabetes onset) (Figure 4A). Blood glucose levels were measured weekly, and mice with glucose levels greater than 200mg/dl on two consecutive days were considered diabetic (Figure 4B). Serum levels of insulin, C-peptide and glucagon were also assessed (Figure S4A–C).

To assess serum levels of TNFα, IL-1β and IFNγ, we performed a high-sensitivity cytokine multiplex assay and observed that the levels of all three cytokines peaked prior to the onset of diabetes and decreased at the established diabetes stage (Figure 4C). Levels of eosinophil chemoattractant, Eotaxin (Garcia-Zepeda et al., 1996) and cytokine, IL-6, were also highest before the onset of diabetes (Figures S4D and S4E). Even though the serum levels of IFNγ have previously been reported to gradually increase before diabetes onset (Schloot et al., 2002), our study has observed a decrease in serum cytokine levels after diabetes is established (5–6 weeks post diabetes). In addition, we confirmed immune cell infiltration in the pancreas by H&E staining and quantified insulitis scores. These results demonstrate that pancreatic insulitis commences prior to the onset of hyperglycemia and decreases at the established diabetes stage (Figures 4D and 4D′).

We then assessed ductal cell proliferation at all stages of diabetes progression by immunofluorescence analyses. Our study demonstrates that ductal cell proliferation (BrdU/DBA double+ cells) peaks at diabetes onset and significantly decreases when diabetes is established (Figures 4E and 4E′), correlating with pancreatic insulitis. Interestingly, immunofluorescence analyses also revealed the presence of NGN3/DBA and pSTAT3/DBA double+ cells in NOD mice before the onset of hyperglycemia, correlating with the increased serum proinflammatory cytokine levels and the development of pancreatic immune cell infiltration observed at pre-diabetic stages (Figure 4F–F′ and 4G–G′). Although some insulin/DBA double+ were detected in the pre-diabetic and new-onset diabetes stages,

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no insulin+ ductal cells were found in the established diabetes stage despite the presence of NGN3 (Figure 4F–F′ and S4F). These observations suggest that the prolonged effects of inflammatory stress in the established diabetes stage may maintain cells in an undifferentiated state and limit the formation of insulin$^+$ ductal cells.

To control for potential effects of aging in a non-NOD mouse model, we used C57BL/6 mice at 3, 8 and 20 weeks of age. Immunofluorescence analyses did not reveal a significant increase in ductal cell proliferation. On the contrary, we observed a significant decrease in BrdU/DBA double+ cells, suggesting an attenuation in ductal cell proliferation with age (Figures S4G and S4H). Moreover, and as expected, H&E staining did not reveal insulitis in these mice, and we did not detect NGN3/DBA or pSTAT3/DBA double+ cells at any of the time points studied (data not shown). Together, our data suggest that ductal cell proliferation and the emergence of NGN3$^+$ or pSTAT3$^+$ ductal cells are driven by insulitis and elevated cytokine levels but not hyperglycemia or age.

DISCUSSION

Diabetes is associated with reduced pancreatic beta cell mass that has prompted major efforts towards the development of novel beta cell regeneration strategies. Under diverse states of physiological stress (high nutrient/caloric intake or inflammation) (Ninov et al., 2013), pathophysiological insults (insulin resistance) (Mezza et al., 2014) or severe pancreatic injury (partial pancreatectomy or duct ligation) (Li et al., 2010; Walker et al., 1992), several studies demonstrate the ability of the pancreas to undergo cellular interconversions. For example, metabolic stress in islets of several type 2 diabetes (T2D) mouse models has been shown to activate NGN3 in pancreatic beta cells, prompting their regression into an endocrine progenitor stage (Talchai et al., 2012). Relief from hyperglycemic stress via insulin therapy allows these insulin$^-$/NGN3$^+$ progenitor cells to redifferentiate into functional insulin$^+$ cells (Wang et al., 2014). In addition, it has been demonstrated that stress encountered during human islet isolation stimulates inflammatory signaling that leads to beta cell dedifferentiation via the emergence of progenitor-specific transcription factors (Negi et al., 2012).

Due to its profusion, exocrine tissue is a strong endogenous candidate for beta cell neogenesis. Previous studies have shown that inflammation can induce exocrine acinar cells to give rise to progenitor cells in cases of autoimmune or chronic pancreatitis in humans (Ko et al., 2013). More recently, Baeyens et al. (2014) used diabetic mice in an elegant study to demonstrate that acinar cells are capable of restoring a glucose-responsive, functional beta cell mass upon a transient cytokine (EGF and CNTF) exposure through the activation of STAT3 and NGN3 (Baeyens et al., 2014).

The exocrine ductal cell is another highly studied pancreatic cell type that has been shown to exhibit cellular plasticity. Using mice, various groups have reported that pancreatic ductal cells can serve as a source of NGN3$^+$ endocrine progenitors following stressors, such as pancreatic injury by duct ligation (Bonner-Weir et al., 1993), partial pancreatectomy (Walker et al., 1992), or targeted beta cell destruction via constitutive IFNγ expression (Gu et al., 1995a; Gu et al., 1995b; Gu and Sarvetnick, 1993). Using human primary ductal cells,

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Seung Kim's group demonstrated that the transgenic expression of NGN3 along with other critical endocrine factors is important for their conversion into glucose-responsive, insulin-secreting endocrine cells (Lee et al., 2013). In addition, some studies have shown that humans that undergo physiological stress in the form of insulin resistance exhibit an increase in Insulin^{+} ductal cells relative to control subjects (Mezza et al., 2014).

Based on these studies, the aim of our work was to investigate whether stress caused specifically by proinflammatory cytokine insults could induce ductal-to-endocrine cell differentiation. In this report, we demonstrate that a cocktail of proinflammatory cytokines (TNFα, IL-1β, IFNγ) can activate the endocrine program in exocrine ductal cells. While previous studies have reported the effect of chronic inflammatory cytokine stimulation on mouse ductal cells in vivo (Gu et al., 1997; Gu et al., 1995a; Gu et al., 1995b), our study is unique for several reasons. First, we report that acute proinflammatory cytokine stimulation via pancreatic intraductal injection is able to induce an increase in ductal cell proliferation in vivo as measured by BrdU incorporation. The decrease, rather than increase, in ductal cell proliferation observed in vitro could be potentially due to the direct and sustained exposure of the target cells to inflammatory cytokines that may not be observed in vivo. Second, the significant increase in insulin^{+} ductal cells in our intraductal injection model suggests that an acute insult of proinflammatory cytokines is sufficient to activate ductal-to-endocrine cell reprogramming. Third, the co-expression of insulin with proinsulin and Chromogranin A in ductal cells indicates that they possess characteristics of true beta cells.

By following the progression of diabetes in a chronic model of autoimmune diabetes, the NOD mouse, we observed that ductal cell proliferation and the emergence of NGN3^{+} and pSTAT3^{+} ductal cells correlate with the presence of serum cytokine levels and pancreatic insulitis, which begin prior to the onset of diabetes. Together, our intraductal injection and NOD mouse diabetes progression studies highlight the importance and specificity of proinflammatory cytokines in this phenomenon, and importantly, rule out potential contributions of hyperglycemic stress. Indeed, the detrimental effects of proinflammatory cytokines on beta cells and the pathogenesis of diabetes have been widely documented (Kikodze et al., 2013; Rabinovitch and Suarez-Pinzon, 2003). However, immune cell-derived inflammatory cytokines have also been reported to stimulate beta cell proliferation in mice (Dirice et al., 2014; Dirice and Kulkarni, 2016). In addition, the Joslin Medalist Study, which consists of patients who have had autoimmune diabetes for at least 50 years, has also reported the presence of scattered insulin^{+} cells within the pancreatic exocrine tissue of all subjects after postmortem analyses (Keenan et al., 2010). One possible explanation for these findings is that inflammatory signaling activates beta cell neogenesis from both beta and non-beta cell sources.

Finally, our in vitro cytokine stimulation model provides a mechanistic insight into cytokine-induced endocrine activation. As previously demonstrated, the transgenic expression of key endocrine transcription factor, NGN3, via adenovirus administration is critical for the generation and expansion of insulin-secreting beta cells from human primary ductal cells (Lee et al., 2013). Here, we demonstrate that proinflammatory cytokines can induce the expression of NGN3. While NGN3 expression has been shown to be mediated through STAT3 activation in vitro (Baeyens et al., 2006; Kaucher et al., 2012; Lemper et al., 2015)

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and in vivo (Baeyens et al., 2014) using rodent acinar cells, our study now highlights the relevance of STAT3 and NGN3 in the context of a human ductal cell line, in vitro, and throughout the progression of diabetes in mouse ductal cells, in vivo.

The field of pancreatic plasticity is a dynamic and timely area of investigation that has been the focus of intense examination in recent years (Valdez et al., 2015). In this study, we have investigated the effect(s) of proinflammatory cytokine-induced stress on the differentiation potential of human and mouse ductal cells. Since TNFα, IL-1β, and IFNγ are three of the most critical proinflammatory cytokines implicated in the pathogenesis of type 1 and 2 diabetes (Al-Shukaili et al., 2013; Spranger et al., 2003; Wang et al., 2010), our study now provides some mechanistic insights into a naturally occurring phenomenon. These findings warrant further investigation into the field of cytokine-driven exocrine ductal cell reprogramming, an area that holds translational relevance for the fields of diabetes and pancreatic regenerative medicine.

EXPERIMENTAL PROCEDURES

Cell culture and in vitro stimulations

The human pancreatic ductal epithelial carcinoma cell line, PANC-1, was maintained in DMEM medium supplemented with 10% FBS. Cytokine stimulations were carried out using this medium. For all experiments, PANC-1 cells were plated at $1x10^6$ cells/well of a 12-well plate, and each condition was carried out in triplicate. All experiments were performed a minimum of two times.

Cytokine Stimulations—Cells were exposed to individual or combinations of TNFα, IL-1β, and IFNγ in dose-response and time-course experiments. TNFα [50ng/mL]; IL-1β [25ng/mL]; IFNγ [100ng/mL] corresponds to [1X] concentration.

Cytokine Receptor Antagonist Stimulations—Human recombinant interleukin-1 receptor antagonist (Creative Biomart®, Catalog No. IL1RN-05H) concentration [50μm]; Human recombinant IFNγ receptor antagonist (Sigma-Aldrich®, Catalog No. 15152) concentration [25μM]; Human TNFα receptor antagonist (Santa CruzR, Catalog No. R-7050) concentration [25μM]. Cells were treated with a cocktail of these cytokine receptor antagonists for 48hrs.

STATTIC Stimulations—pSTAT3 inhibitor STATTIC (Selleck Chemicals LLC, Catalog No. S7024) was used at a concentration of 5uM for 48hrs.

Immunofluorescence studies

Immunocytochemistry—Cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature, blocked with 5% donkey serum in PBS triton X (0.1%) for 1hr at room temperature, and stained with primary antibodies (Supplemental Table S1) and appropriate secondary antibodies. Nuclei were stained with DAPI. At least 1,000 cells were counted per well or animal.

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Immunohistochemistry—Pancreases were harvested, fixed with 4% paraformaldehyde, and embedded in paraffin. Sections were stained using primary antibodies (Supplemental Table S1) and appropriate secondary antibodies. Nuclei were stained with DAPI. At least 1,000 cells were counted per well or animal.

qPCR

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's recommendations followed by cDNA synthesis using standard RT-PCR protocols. Approximately 25ng of cDNA template was mixed with 2x Sybr Green PCR Master Mix (BioRad) and diluted to 1x with 100nM primers (Supplemental Table S2) and dH_2O.

Immunoblot analyses

Methods for immunoblot analyses have been described previously (Bhatt et al., 2015).

BrdU administration

Intraperitoneal injections of BrdU were performed in all mice 3 days and 5 hours prior to sacrifice at a concentration of [100mg/kg BW] (El Ouaamari et al., 2013).

Pancreatic intraductal injections

For this experiment, 8–10 week old male C57BL/6 mice were anesthetized with Avertin (240 mg/kg intraperitoneally). An incision of 3 cm was performed through the center of the abdomen. Then the duodenum was exposed and the common bile duct was clamped near the liver. The intraductal injection was performed using a 28G1/2 U-100 Insulin Syringe into the pancreatic duct from the duodenum through the ampulla of Vater. The needle was clamped and 150 μl of saline or a single dose of a cocktail of proinflammatory cytokines ([1X] = TNFα [50ng/mL]; IL-1β [25ng/mL]; IFNγ [100ng/mL], n=3), ([2X] = TNFα [100ng/mL]; IL-1β [50ng/mL]; IFNγ [200ng/mL], n=6), ([10X] = TNFα [500ng/mL]; IL-1β [250ng/mL]; IFNγ [1000ng/mL], n=4) or saline injections (n=5) were administered. After clamp removal, the wound on the ampulla was sealed with 3M Vetbond Tissue Adhesive (3M Animal Care). Abdominal muscle layer was closed with interrupted suture and the overlying skin was sutured using Needle Dafilon® and Polyamide Monofilament (B. Braun). Blood glucose levels were measured once a week in the morning for 3 weeks. Pancreata were harvested 3 weeks post-injection.

NOD mouse diabetes progression studies

Female NOD mice were purchased from The Jackson Laboratory (Farmington, CT USA) and maintained in a pathogen free animal facility at Joslin Diabetes Center. Diabetes was tracked by measuring their morning blood glucose levels suing a Contour® Blood Glucose Meter (Bayer) once a week every week. Mice were considered diabetic after two consecutive days of blood glucose levels being greater than 200mg/dl (Dirice et al., 2014). A total of n=4–9 mice per group were used and divided into 5 groups: 3 weeks (n=5), 8 weeks (n=5) and 12 weeks of age (n=4), new onset diabetes (1–2 weeks after onset; n=9) and established diabetes (5–6 weeks after onset; n=8). The pancreases of these animals were harvested and sectioned for immunohistochemical analyses (Dirice et al., 2014).

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Insulitis scores

Pancreatic sections of NOD mice were stained with H&E were ranked for insulitis using the following parameters: 0 = normal islet morphology with no insulitis, 1 = peri-insulitis, 2 = insulitis, and 3 = islet remnant as reported previously (Dirice et al., 2014).

Serum Cytokine Measurements using Multiplex Assay

We collected blood samples from heart puncture of all NOD mice and obtained serum. Samples were stored at −70° C until analysis. Serum cytokine levels were measured by Luminex bead-based multiplex assay using a dual-laser Luminex-100 instrument (Millipore) by the Joslin Diabetes Center Assay Core.

Statistical analyses

Data are expressed as mean ± SEM and a two-tailed Student t test or ANOVA was used to confirm significance at p 0.05.

Study approval

All animal experiments were conducted after approval by the Institutional Animal Care and Use Committee (IACUC) of the Joslin Diabetes Center in accordance with National Institutes of Health (NIH) guidelines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES AND NOTES

Al-Shukaili A, Al-Ghafri S, Al-Marhoobi S, Al-Abri S, Al-Lawati J, Al-Maskari M. Analysis of inflammatory mediators in type 2 diabetes patients. International journal of endocrinology. 2013; 2013:976810. [PubMed: 23762057]

Attali M, Stetsyuk V, Basmaciogullari A, Aiello V, Zanta-Boussif MA, Duvillie B, Scharfmann R. Control of beta-cell differentiation by the pancreatic mesenchyme. Diabetes. 2007; 56:1248–1258. [PubMed: 17322477]

Baeyens L, Bonne S, German MS, Ravassard P, Heimberg H, Bouwens L. Ngn3 expression during postnatal in vitro beta cell neogenesis induced by the JAK/STAT pathway. Cell death and differentiation. 2006; 13:1892–1899. [PubMed: 16514419]

Baeyens L, Lemper M, Leuckx G, De Groef S, Bonfanti P, Stange G, Shemer R, Nord C, Scheel DW, Pan FC, et al. Transient cytokine treatment induces acinar cell reprogramming and regenerates functional beta cell mass in diabetic mice. Nature biotechnology. 2014; 32:76–83.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Bhatt S, Gupta MK, Khamaisi M, Martinez R, Gritsenko MA, Wagner BK, Guye P, Busskamp V, Shirakawa J, Wu G, et al. Preserved DNA Damage Checkpoint Pathway Protects against Complications in Long-Standing Type 1 Diabetes. Cell metabolism. 2015; 22:239–252. [PubMed: 26244933]

Bonner-Weir S, Baxter LA, Schuppin GT, Smith FE. A second pathway for regeneration of adult exocrine and endocrine pancreas. A possible recapitulation of embryonic development. Diabetes. 1993; 42:1715–1720. [PubMed: 8243817]

Brewington R, Chatterji M, Zoubine M, Miranda RN, Norimatsu M, Shnyra A. IFN-gamma-independent autocrine cytokine regulatory mechanism in reprogramming of macrophage responses to bacterial lipopolysaccharide. J Immunol. 2001; 167:392–398. [PubMed: 11418675]

de la Rosa EJ, de Pablo F. Proinsulin: from hormonal precursor to neuroprotective factor. Frontiers in molecular neuroscience. 2011; 4:20. [PubMed: 21949502]

Dirice E, Kahraman S, Jiang W, El Ouaamari A, De Jesus DF, Teo AK, Hu J, Kawamori D, Gaglia JL, Mathis D, et al. Soluble factors secreted by T cells promote beta-cell proliferation. Diabetes. 2014; 63:188–202. [PubMed: 24089508]

Dirice E, Kulkarni RN. Harnessing immune cells to enhance beta-cell mass in type 1 diabetes. Journal of investigative medicine : the official publication of the American Federation for Clinical Research. 2016; 64:14–20. [PubMed: 26755809]

Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature. 2004; 429:41–46. [PubMed: 15129273]

El Ouaamari A, Kawamori D, Dirice E, Liew CW, Shadrach JL, Hu J, Katsuta H, Hollister-Lock J, Qian WJ, Wagers AJ, et al. Liver-derived systemic factors drive beta cell hyperplasia in insulin-resistant states. Cell reports. 2013; 3:401–410. [PubMed: 23375376]

Garcia-Zepeda EA, Rothenberg ME, Ownbey RT, Celestin J, Leder P, Luster AD. Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. Nature medicine. 1996; 2:449–456.

Gingold H, Tehler D, Christoffersen NR, Nielsen MM, Asmar F, Kooistra SM, Christophersen NS, Christensen LL, Borre M, Sorensen KD, et al. A dual program for translation regulation in cellular proliferation and differentiation. Cell. 2014; 158:1281–1292. [PubMed: 25215487]

Gu D, Arnush M, Sarvetnick N. Endocrine/exocrine intermediate cells in streptozotocin-treated Ins-IFN-gamma transgenic mice. Pancreas. 1997; 15:246–250. [PubMed: 9336787]

Gu D, Arnush M, Sawyer SP, Sarvetnick N. Transgenic mice expressing IFN-gamma in pancreatic beta-cells are resistant to streptozotocin-induced diabetes. The American journal of physiology. 1995a; 269:E1089–1094. [PubMed: 8572201]

Gu D, Molony L, Krahl T, Sarvetnick N. Treatment of IFN-gamma transgenic mice with anti-IFN-gamma reveals the remodeling capacity of the adult pancreas. Diabetes. 1995b; 44:1161–1164. [PubMed: 7556951]

Gu D, Sarvetnick N. Epithelial cell proliferation and islet neogenesis in IFN-g transgenic mice. Development. 1993; 118:33–46. [PubMed: 8104143]

Hardikar AA, Marcus-Samuels B, Geras-Raaka E, Raaka BM, Gershengorn MC. Human pancreatic precursor cells secrete FGF2 to stimulate clustering into hormone-expressing islet-like cell aggregates. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100:7117–7122. [PubMed: 12799459]

Ivashkiv LB, Donlin LT. Regulation of type I interferon responses. Nature reviews Immunology. 2014; 14:36–49.

Kaucher AV, Oatley MJ, Oatley JM. NEUROG3 is a critical downstream effector for STAT3-regulated differentiation of mammalian stem and progenitor spermatogonia. Biology of reproduction. 2012; 86:164, 161–111. [PubMed: 22378757]

Keenan HA, Sun JK, Levine J, Doria A, Aiello LP, Eisenbarth G, Bonner-Weir S, King GL. Residual insulin production and pancreatic ss-cell turnover after 50 years of diabetes: Joslin Medalist Study. Diabetes. 2010; 59:2846–2853. [PubMed: 20699420]

Kikodze N, Pantsulaia I, Rekhviashvili K, Iobadze M, Dzhakhutashvili N, Pantsulaia N, Kukuladze N, Bikashvili N, Metreveli D, Chikovani T. Cytokines and T regulatory cells in the pathogenesis of type 1 diabetes. Georgian medical news. 2013:29–35. [PubMed: 24099812]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Kim YH, Larsen HL, Rue P, Lemaire LA, Ferrer J, Grapin-Botton A. Cell cycle-dependent differentiation dynamics balances growth and endocrine differentiation in the pancreas. PLoS biology. 2015; 13:e1002111. [PubMed: 25786211]

Ko SB, Azuma S, Yokoyama Y, Yamamoto A, Kyokane K, Niida S, Ishiguro H, Ko MS. Inflammation increases cells expressing ZSCAN4 and progenitor cell markers in the adult pancreas. American journal of physiology Gastrointestinal and liver physiology. 2013; 304:G1103–1116. [PubMed: 23599043]

Kopp JL, Dubois CL, Schaffer AE, Hao E, Shih HP, Seymour PA, Ma J, Sander M. Sox9+ ductal cells are multipotent progenitors throughout development but do not produce new endocrine cells in the normal or injured adult pancreas. Development. 2011; 138:653–665. [PubMed: 21266405]

Kulkarni RN, Jhala US, Winnay JN, Krajewski S, Montminy M, Kahn CR. PDX-1 haploinsufficiency limits the compensatory islet hyperplasia that occurs in response to insulin resistance. The Journal of clinical investigation. 2004; 114:828–836. [PubMed: 15372107]

Lee J, Sugiyama T, Liu Y, Wang J, Gu X, Lei J, Markmann JF, Miyazaki S, Miyazaki J, Szot GL, et al. Expansion and conversion of human pancreatic ductal cells into insulin-secreting endocrine cells. eLife. 2013; 2:e00940. [PubMed: 24252877]

Lefebvre B, Belaich S, Longue J, Vandewalle B, Oberholzer J, Gmyr V, Pattou F, Kerr-Conte J. 5′-AZA induces Ngn3 expression and endocrine differentiation in the PANC-1 human ductal cell line. Biochemical and biophysical research communications. 2010; 391:305–309. [PubMed: 19913512]

Lemper M, Leuckx G, Heremans Y, German MS, Heimberg H, Bouwens L, Baeyens L. Reprogramming of human pancreatic exocrine cells to beta-like cells. Cell death and differentiation. 2015; 22:1117–1130. [PubMed: 25476775]

Li WC, Rukstalis JM, Nishimura W, Tchipashvili V, Habener JF, Sharma A, Bonner-Weir S. Activation of pancreatic-duct-derived progenitor cells during pancreas regeneration in adult rats. Journal of cell science. 2010; 123:2792–2802. [PubMed: 20663919]

Lieber M, Mazzetta J, Nelson-Rees W, Kaplan M, Todaro G. Establishment of a continuous tumor-cell line (panc-1) from a human carcinoma of the exocrine pancreas. International journal of cancer Journal international du cancer. 1975; 15:741–747. [PubMed: 1140870]

Lukinius A, Stridsberg M, Wilander E. Cellular expression and specific intragranular localization of chromogranin A, chromogranin B, and synaptophysin during ontogeny of pancreatic islet cells: an ultrastructural study. Pancreas. 2003; 27:38–46. [PubMed: 12826904]

Mezza T, Muscogiuri G, Sorice GP, Clemente G, Hu J, Pontecorvi A, Holst JJ, Giaccari A, Kulkarni RN. Insulin resistance alters islet morphology in nondiabetic humans. Diabetes. 2014; 63:994–1007. [PubMed: 24215793]

Negi S, Jetha A, Aikin R, Hasilo C, Sladek R, Paraskevas S. Analysis of beta-cell gene expression reveals inflammatory signaling and evidence of dedifferentiation following human islet isolation and culture. PloS one. 2012; 7:e30415. [PubMed: 22299040]

Ninov N, Hesselson D, Gut P, Zhou A, Fidelin K, Stainier DY. Metabolic regulation of cellular plasticity in the pancreas. Current biology : CB. 2013; 23:1242–1250. [PubMed: 23791726]

Nir T, Melton DA, Dor Y. Recovery from diabetes in mice by beta cell regeneration. The Journal of clinical investigation. 2007; 117:2553–2561. [PubMed: 17786244]

O'Reilly LA, Gu D, Sarvetnick N, Edlund H, Phillips JM, Fulford T, Cooke A. alpha-Cell neogenesis in an animal model of IDDM. Diabetes. 1997; 46:599–606. [PubMed: 9075799]

Rabinovitch A, Suarez-Pinzon WL. Role of cytokines in the pathogenesis of autoimmune diabetes mellitus. Reviews in endocrine & metabolic disorders. 2003; 4:291–299. [PubMed: 14501180]

Ramiya VK, Maraist M, Arfors KE, Schatz DA, Peck AB, Cornelius JG. Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. Nature medicine. 2000; 6:278–282.

Rukstalis JM, Habener JF. Snail2, a mediator of epithelial-mesenchymal transitions, expressed in progenitor cells of the developing endocrine pancreas. Gene expression patterns : GEP. 2007; 7:471–479. [PubMed: 17185046]

Rukstalis JM, Habener JF. Neurogenin3: a master regulator of pancreatic islet differentiation and regeneration. Islets. 2009; 1:177–184. [PubMed: 21099270]

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Schloot NC, Hanifi-Moghaddam P, Goebel C, Shatavi SV, Flohe S, Kolb H, Rothe H. Serum IFN-gamma and IL-10 levels are associated with disease progression in non-obese diabetic mice. Diabetes/metabolism research and reviews. 2002; 18:64–70. [PubMed: 11921420]

Solar M, Cardalda C, Houbracken I, Martin M, Maestro MA, De Medts N, Xu X, Grau V, Heimberg H, Bouwens L, et al. Pancreatic exocrine duct cells give rise to insulin-producing beta cells during embryogenesis but not after birth. Developmental cell. 2009; 17:849–860. [PubMed: 20059954]

Spranger J, Kroke A, Mohlig M, Hoffmann K, Bergmann MM, Ristow M, Boeing H, Pfeiffer AF. Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. Diabetes. 2003; 52:812–817. [PubMed: 12606524]

Talchai C, Xuan S, Lin HV, Sussel L, Accili D. Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure. Cell. 2012; 150:1223–1234. [PubMed: 22980982]

Valdez IA, Teo AK, Kulkarni RN. Cellular stress drives pancreatic plasticity. Science translational medicine. 2015; 7:273ps272.

Walker NI, Winterford CM, Kerr JF. Ultrastructure of the rat pancreas after experimental duct ligation. II. Duct and stromal cell proliferation, differentiation, and deletion. Pancreas. 1992; 7:420–434. [PubMed: 1641387]

Wang C, Guan Y, Yang J. Cytokines in the Progression of Pancreatic beta-Cell Dysfunction. International journal of endocrinology. 2010; 2010:515136. [PubMed: 21113299]

Wang Z, York NW, Nichols CG, Remedi MS. Pancreatic beta cell dedifferentiation in diabetes and redifferentiation following insulin therapy. Cell metabolism. 2014; 19:872–882. [PubMed: 24746806]

Wu Y, Li J, Saleem S, Yee SP, Hardikar AA, Wang R. c-Kit and stem cell factor regulate PANC-1 cell differentiation into insulin- and glucagon-producing cells. Laboratory investigation; a journal of technical methods and pathology. 2010; 90:1373–1384.

Xiao X, Chen Z, Shiota C, Prasadan K, Guo P, El-Gohary Y, Paredes J, Welsh C, Wiersch J, Gittes GK. No evidence for beta cell neogenesis in murine adult pancreas. The Journal of clinical investigation. 2013; 123:2207–2217. [PubMed: 23619362]

Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nature reviews Cancer. 2009; 9:798–809. [PubMed: 19851315]

Zhu L, Skoultchi AI. Coordinating cell proliferation and differentiation. Current opinion in genetics & development. 2001; 11:91–97. [PubMed: 11163157]

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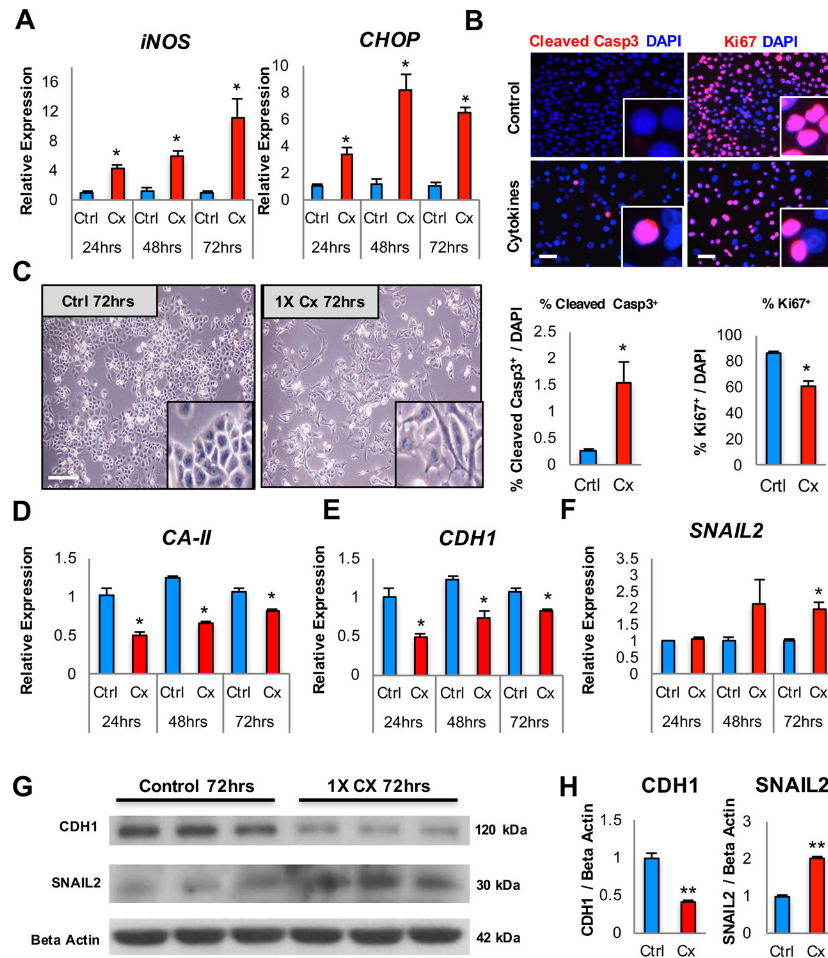

Figure 1. Epithelial-to-Mesenchymal Transition in cytokine-treated human ductal cells

(A) qPCR analysis of *iNOS* and *CHOP* in untreated (Ctrl) or cytokine-treated (1X Cx: TNFα [50ng/mL]; IL-1β [25ng/mL]; IFNγ [100ng/mL]) PANC-1 cells.

(B) Representative immunofluorescence images of untreated or cytokine-treated cells for Ki67 and cleaved caspase-3 staining at 72hrs (top). The quantification of Ki67 or cleaved caspase-3 / DAPI data are shown in the bottom panels. Scale bar = 100μM.

(C) Representative brightfield images of PANC-1 cells at 72hrs under untreated or cytokine-treated conditions. Scale = 100μM.

(D–F) qPCR analysis of (D) ductal marker, *CA-II*; (E) epithelial marker, *CDH1*; and (F) mesenchymal marker, *SNAIL2*, in untreated or cytokine-treated cells at 24hrs, 48hrs and 72hrs.

(G, H) Protein immunoblot analysis (G) and quantification (H) of CDH1 (E-cadherin) and SNAIL2 after 1X Cx treatment at 72hrs. All qPCR data was normalized to beta actin. Data represent mean ± SEM. *p 0.05 and **p 0.005 relative to untreated controls. (n=3 for each group). All experiments were repeated at least on two independent occasions.

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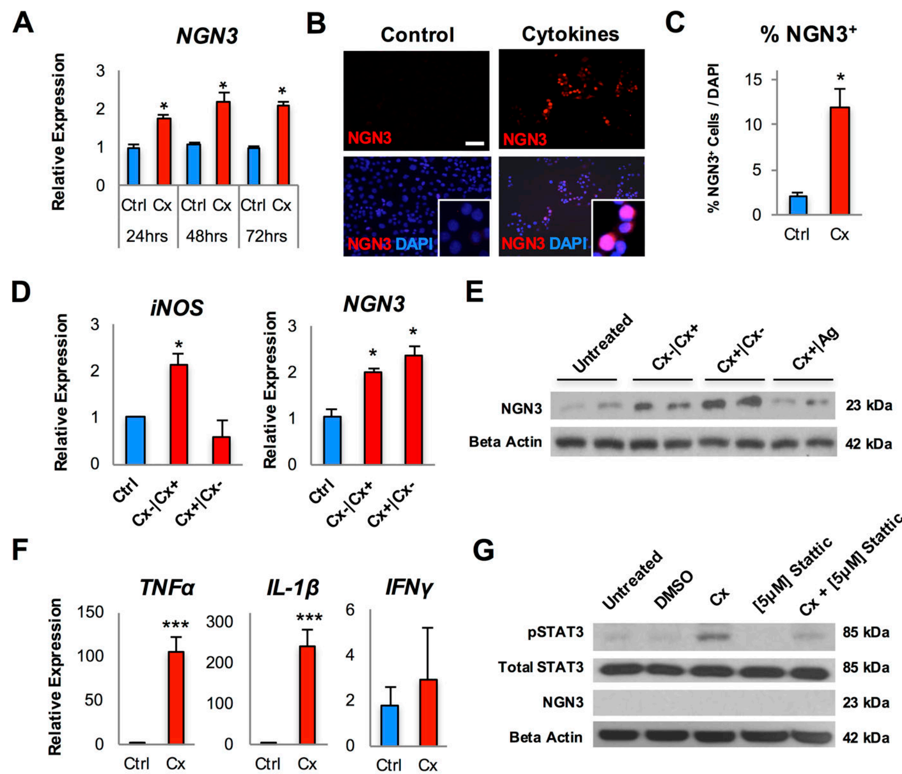

Figure 2. NGN3 upregulation in human ductal cells upon inflammatory cytokine stimulation

(A) qPCR analysis of *NGN3* in untreated or cytokine-treated (TNFα, IL-1β and IFNγ) PANC-1 cells at 24hrs, 48hrs and 72hrs.

(B, C) Representative immunofluorescence images (B) and quantification (C) of untreated or cytokine-treated cells for NGN3 (red) and DAPI (blue) at 72hrs.

(D) qPCR analysis of i*NOS* and *NGN3* after 1) 96hrs untreated conditions (ctrl), 2) 48hr without cytokines + 48hr with cytokines (Cx−|Cx+), or 3) 48hrs with cytokines + 48hrs of cytokine removal (Cx+|Cx−).

(E) Protein immunoblot analysis of NGN3 after 1) 96hrs untreated conditions, 2) 48hr without cytokines + 48hr with cytokines (Cx−|Cx+), 3) 48hrs with cytokines + 48hrs of cytokine removal (Cx+|Cx−) and 4) 48hrs with cytokines + 48hrs of cytokine removal with specific cytokine receptor antagonists (*TNFα R* [25µM]; IL-1 R [50µM]; IFN*γ* R [25µM]; (Cx+|Ag). Beta actin was used as a loading control.

(F) qPCR analysis of *TNFα*, *IL-1β*, and *IFNγ* in untreated and [1X] cytokine-treated PANC-1 cells at 48hrs. All qPCR data was normalized to beta actin.

(G) Protein immunoblot analysis of pSTAT3, total STAT3, and NGN3, in untreated control, DMSO-treated control, 1X cytokine-treated (Cx), Stattic [5µM], or [1X] Cytokine + Stattic [5µM] treatment after 48hrs. Beta actin was used as a loading control.

Data represent mean ± SEM. *p 0.05 and ***p 0.0005 (n=3 for each group). Experiments were repeated at least on two independent occasions.

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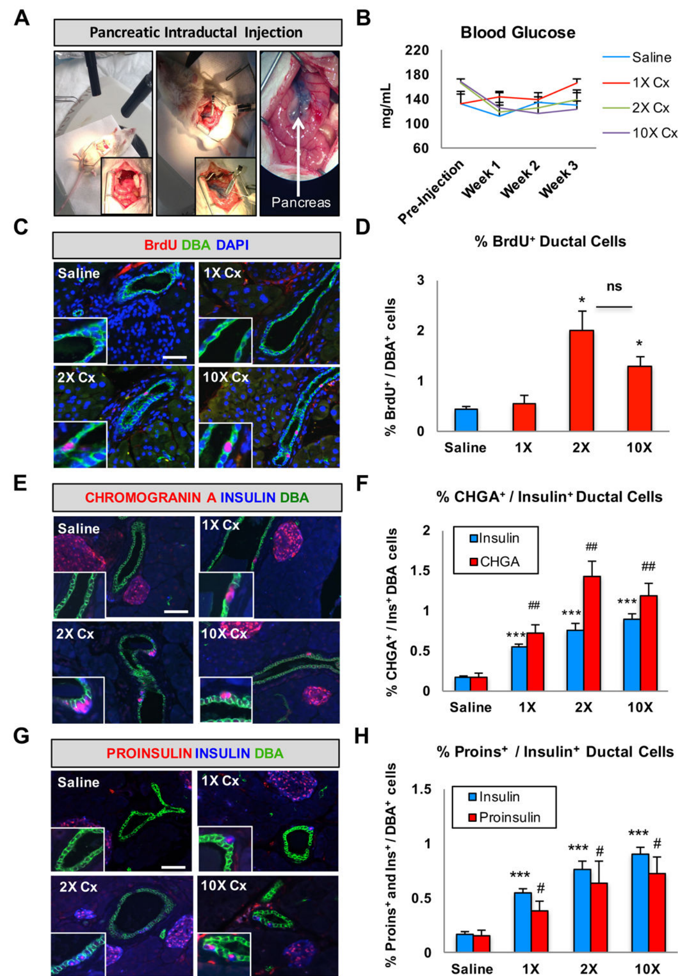

Figure 3. Pancreatic intraductal injection of proinflammatory cytokines stimulates ductal cell proliferation and an increase in insulin$^+$ ductal cells in mice

(A) Representative image of mouse undergoing pancreatic intraductal injection surgery. Evans blue dye shows inflated pancreas. Intrapancreatic injections into the pancreatic duct of C57BL/6 (B6) mice aged 8–10 weeks were performed using saline or a single injection of the cocktail of inflammatory cytokines (TNFα, IL-1β, IFNγ) at [1X] [2X] and [10X] doses. 3 weeks after injection, the pancreas was harvested. Mice were injected intraperitoneally with [100mg/kg BW] of BrdU 3 days and 5 hours before harvesting pancreas.

(B) Blood glucose levels of saline, [1X], [2X] or [10X] cytokine injected mice.

(C, D) Representative immunofluorescence images (C) and quantification (D) of saline injected, [1X], [2X] or [10X] cytokine injected mice for BrdU (red) and DBA (green) at 3 weeks post-injection DAPI was stained with blue. Sale bar = 100μM.

(E, F) Representative immunofluorescence images (E) and quantification (F) of saline injected, [1X], [2X] or [10X] cytokine injected mice for Chromogranin A (CHGA) (red), Insulin (blue) and DBA (green) at 3 weeks post-injection. Scale bar = 100μM.

(G, H) Immunofluorescence images (G) and quantification (H) of saline injected, [1X], [2X] or [10X] cytokine injected mice for Proinsulin (red), Insulin (blue), and DBA (green) at 3 weeks post-injection. Scale bar = 100μM.

Data represent mean ± SEM. *p 0.05, ## and **p 0.005, ***p 0.0005 relative to respective saline injected controls. (n=4–6 for each group).

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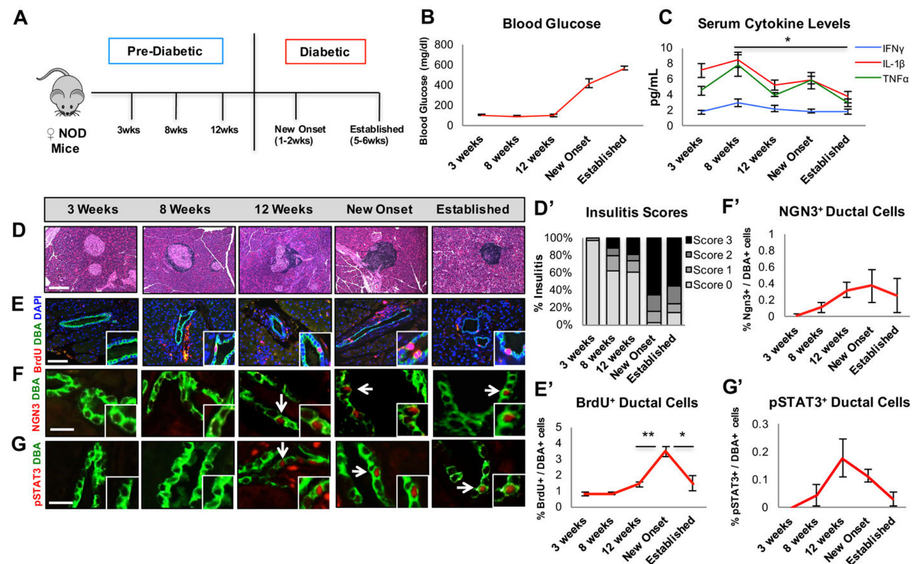

Figure 4. Ductal cell proliferation and emergence of NGN3$^+$ and pSTAT3$^+$ ductal cells correlate with immune cell infiltration, and not hyperglycemia, in NOD mice

(A) Experimental design using female NOD mouse model of autoimmune diabetes.

(B) Blood glucose levels of NOD mice throughout diabetes progression.

(C) Serum levels of TNFα, IL-1β and IFNγ cytokines measured by Luminex multiplex assay. * applies to the three cytokines.

(D, D′) Representative brightfield images (D) and quantification (D′) of islet insulitis at different time points of diabetes progression. Scale bar = 100μM.

(E, E′) Representative immunofluorescence images (E) and quantification (E′) of BrdU$^+$ (red) ductal (DBA$^+$ green) cells in NOD mice throughout diabetes progression. Nuclei were stained with DAPI (Blue). Scale bar = 100μM.

(F, F′) Representative immunofluorescence images (F) and quantification (F′) of NGN3$^+$ (red) ductal (DBA; green) cells in NOD mice throughout diabetes progression. Scale = 20μM.

(G, G′) Representative immunofluorescence images (G) and quantification (G′) of pSTAT3$^+$ (red) ductal (DBA; green) cells in NOD mice throughout diabetes progression. Scale = 20μM.

Data represent mean ± SEM. *p 0.05 **p 0.005 (n=4–9 for each group).