Inhibition of TGFβ Signaling Does Not Improve the Limited Proliferative Response to Nkx6.1 Or Pdx-1 Overexpression in Aged Rat Islets

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Thesis submitted in partial fulfillment of
the requirements for the degree of Master of Science in the Department of
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ABSTRACT

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Abstract

A deficiency in functional pancreatic β-cells is a defining feature in type 1 and type 2 diabetes. The development of therapeutic strategies for replacement and regeneration of β-cell mass is a key objective of current diabetes research. The Newgard lab has had a particular focus in exploring novel β-cell replication pathways in order to identify targets that enhance β-cell proliferation, survival, and function. The β-cell developmental transcription factors Nkx6.1 and Pdx-1 each have a profound proliferative effect when overexpressed in young rat islets in vitro. The unique ability of these factors and the pathways that they control to expand functional β-cell mass while either being neutral or positive for other key functions (survival, insulin secretion) encourages further studies to better elucidate candidate target genes within these pathways.

A major limitation of the research to date is that Nkx6.1 and Pdx-1 only exert their proliferative effects in young (2 months) rodent islets and not in old (8-10 months) rodent islets. Moreover, these factors are only weakly active in human islets, most of which come from middle-aged donors. Nkx6.1 and Pdx-1 engage pathways that are upstream of the core cell cycle machinery and that have the potential to be stimulated in a β-cell specific manner, but use of this approach will depend on a better understanding

of the differences between human and rodent islets, which may be modeled by the differences between old and young rat islets.

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1. Introduction

The islets of Langerhans are clusters of endocrine tissue within the pancreas that include β-cells, the body's main source of insulin. Their purpose is to maintain blood glucose homeostasis by secreting insulin in response to increases in blood glucose and other nutrients and potentiators. Insulin secretion into the bloodstream causes peripheral tissues to increase glucose uptake and metabolism, effectively lowering blood glucose levels after a meal is consumed. Type 1 and Type 2 diabetes have different etiology, but importantly, both are characterized by a deficit in the amount of β-cells needed to normalize blood glucose, resulting in chronic hyperglycemia, which if untreated has widespread destructive effects on multiple organs.

Clinical transplantation trials show that diabetes can be completely reversed by surgically restoring β-cell mass [1,2]. The replacement of β-cell mass by islet transplantation is technically feasible, but limited by the availability of donor tissue and poor graft survival; thus, the development of strategies that preserve an existing donor population of β-cells by enhancing turnover/renewal might improve islet transplantation outcome. Moreover, current transplantation protocols are clinically invasive and use powerful immunosuppressive drugs to prevent donor tissue rejection [2], making pharmacological treatment strategies aimed at restoring a functionally adequate endogenous β-cell mass even more desirable. Unfortunately, their

development is hindered by a lack of knowledge about the molecular mechanisms underlying β-cell replication in humans.

Efforts to uncover such mechanisms have centered on observations from rodent transgenic mouse models, primary cultured cells, and cell lines. The authoritative findings from these efforts are that 1) proliferation is thought to be the primary means of regenerating β-cells [3,4], 2) β-cells can be induced to replicate in the presence of growth factors or under physiological conditions such as obesity or pregnancy that increase the demand for insulin [5], and 3) excessive β-cell proliferation is often accompanied by dedifferentiation and loss of glucose-stimulated insulin secretion (GSIS) [6].

The availability of human islets for research has led to the discovery of intrinsic differences in the proliferative responses of rodent and human β-cells. Specifically, most conditions found to induce rodent β-cell replication are not capable of inducing the same response in human β-cells. For example, human pancreatic tissue pathology studies have not identified evidence of human β-cell proliferation under physiological conditions such as obesity or during pregnancy, both conditions documented as having a positive effect on rodent β-cell proliferation [7-9]. Moreover, the basal rate of proliferation in the adult human pancreas is approximately 10-fold less than that of adult rodents [10-12]. Nevertheless, it is important to note that there is published evidence of human β-cell proliferation in vivo when human islet grafts overexpressing cell cycle activators such as cdk6 or lacking cell cycle repressors such as p57 were

transplanted into nude mice [13-14]. These results suggest that induction of human β-cell proliferation is technically feasible but will require better understanding of the molecular mechanisms responsible for the variable response to proliferative stimuli in human islets compared to rodent islets.

The homeobox transcription factors Pdx-1 and Nkx6.1 are upstream of the core cell cycle machinery and play fundamental roles in the normal development of islet β-cells. Nkx6.1 is required for β-cell development from pancreatic progenitors [15]. It is first expressed broadly in the undifferentiated epithelial cells of the early pancreatic buds. After a wave of differentiation starting at embryonic day 13 called the secondary transition, expression is restricted to a subset of proliferating islet cell progenitors [16]. Nkx6.1 is expressed specifically in differentiated β-cells once development is completed [17]. Nkx6.1 is essential for GSIS and optimal β-cell function [18]. Biochemical and genomic analysis of Nkx6.1 suggest it is both a transcriptional repressor, inhibiting expression of the glucagon gene, as well as a transcriptional activator, positively regulating its own transcription and several cell cycle regulatory genes upon overexpression [19,20].

Pdx-1 plays an early role in pancreas development, as it is essential for the creation of pancreatic precursor cells and the formation of the exocrine and endocrine pancreas [21]. Pdx-1 also assists in the maintenance of the differentiated β-cell phenotype by regulating expression of insulin, Glut2, glucokinase, and islet amyloid

peptide in humans [22]. Loss of function mutations of Pdx-1 in humans causes a form of maturity-onset diabetes of the young, type 4 (MODY4) [23]. Adenovirus-mediated overexpression of Pdx-1 or Nkx6.1 in young rat islets profoundly increases β-cell proliferation in vitro (Figure 3). Overexpression of Nkx6.1 is also able to enhance glucose stimulated insulin secretion (GSIS), while Pdx-1 overexpression maintains GSIS and has an additional anti-apoptotic effect to boost survival [19].

The cdk4/6 inhibitor $p16^{INK4a}$ recently emerged as a key effector of declining islet proliferation with age [28,29]. At the molecular level, cell cycle arrest by the INK ($p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$, and $p19^{Ink4d}$) family of cdk inhibitors is imposed by binding to and inhibiting the catalytic activity of the cdk4/6:cyclin-D complex that is required for hyperphosphorylation and inactivation of the retinoblastoma protein (pRb) [30]. When cdk4/6 partner with cyclin D in early G1 phase of the cell cycle, they promote cell cycle progression by phosphorylating pRb [31]. Upon further phosphorylation by other cyclin:cdk complexes, pRb releases E2F1, initiating a transcriptional program of cyclins, cdks, DNA synthesis genes, and other genes needed to transition through S-phase [32,33].

A 2006 study reported that aged $p16^{INK4a}$ null mice had rates of β-cell proliferation comparable to their wild-type juvenile counterparts [28]. The study also quantified β-cell proliferation in mice transgenic for $p16^{INK4a}$, and found replication rates to be significantly diminished relative to controls. The most profound effect in

transgenic mice was in younger islets, suggesting that an overabundance of $p16^{INK4a}$ is capable of inducing β-cell cycle arrest, and that increased expression with age may cause the reduced basal proliferation rate in aged mice. Hence, the age-dependent rise in $p16^{INK4a}$ expression is thought to be a limiting factor in aged rodent β-cell proliferation.

The Polycomb group (PcG) protein Enhancer of zeste homolog 2 (Ezh2) is a chromatin modifying protein that regulates the expression of genes that control pluripotency, proliferation, stem cell renewal, and tumorigenesis [34]. PcG proteins exist in larger polycomb repressor complexes 1 (PRC1) and PRC2. Ezh2 is the catalytic SET-domain containing histone methyltransferase that marks lysine 27 of histone 3 with 1, 2, or 3 methyl groups (H3K27me3) at the *Ink4a* promoter [35]. H3K27me3 enrichment is historically associated with transcriptional silencing [36]. In mouse and human β-cells, endogenous Ezh2 levels decline with age [37-39]; this temporal loss of Ezh2 expression limits adult β-cell replication through derepression of the *Ink4a* locus [38]. The age-dependent loss of PRC1 and PRC2 presence at the islet *Ink4a* locus is accompanied by increased trithorax group (TrxG) protein complex enrichment **(Figure 1).** The catalytic component of the TrxG complex, Mll1, makes a H3K4me3 chromatin modification associated with transcriptional activation [39].

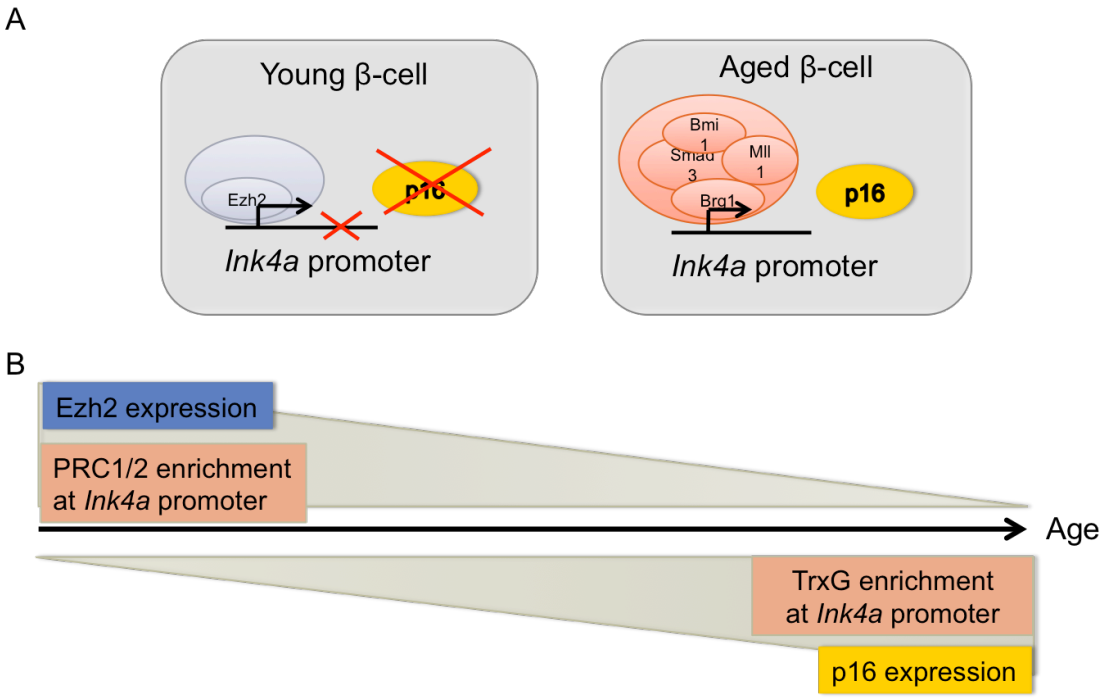

Figure 1: (A) Molecular aging of the Ink4a locus in islets. (B) Summary schematic of epigenetic changes at the Ink4a locus as β-cells age.

A landmark study found that as β-cells age in both rodent and human islets, replication becomes limited by a decline in expression of platelet-derived growth factor receptor (PDGFRα) [37]. PDGFRα signaling controls replication by initiating a signaling cascade that increases Ezh2 expression and represses $p16^{INK4a}$ and $p19^{Arf}$ transcription to promote cell cycle progression. By generating mice with conditional inactivation of Ezh2 in the setting of β-cell specific PDGFRα overexpression, the authors confirmed that Ezh2 is required for PDGF-induced β-cell expansion. Crucially, conditional Ezh2 inactivation in β-cells with activated PDGFRα signaling prevented these changes. This study also

identified PDGFRα as an activator of Erk1/2 signaling in β-cells, which stimulates replication via upregulation of cyclin D and downregulation of $p16^{INK4a}$. Moreover, *in vivo* enhancement of β-cell PDGFRα signaling mitigated the age-dependent regression of β-cell proliferation in mice.

Forced expression of an Ezh2 transgene in aged mice does not repress *Ink4a* transcription, nor does it restore capacity for β-cell replication [39]. Notably, combining knockdown of Mll1 in the context of forced Ezh2 expression represses *Ink4a* transcription and stimulates aged β-cell replication in vitro. Thus, it seems that Ezh2 cannot dislodge the Mll1 complex in older mice, and Ezh2 cannot place the repressive mark unless it is removed [39].

The effects of the TGFβ signaling pathway are known to be highly cell type and context dependent, but the pathway largely exerts its varied effects by regulating gene expression. TGFβ family members regulate gene expression by receptor-mediated activation of SMAD transcription factors. SMADs regulate the transcriptional output of active genes and can also open repressive chromatin [40]. Ligand binding to a TGFβ-Type II receptor recruits and phosphorylates a TGFβ-Type I receptor (TβRI), initiating Smad2/Smad3 complex phosphorylation, formation of Smad2/3-coSmad4 oligomers, and migration to the nucleus where they bind to other DNA-binding transcription factors for target gene recognition and transcriptional regulation [40].

Smad3 is a component of both the TGFβ signaling pathway and the Mll1-TrxG complex [47], so it provides a regulatory link between TGFβ signaling and epigenetic control of the *Ink4a* locus. This premise is based in part on the finding that activation of the TGFβ receptor increases $p16^{Ink4a}$ expression and mimics replicative senescence [47]. We suspect that enhanced TrxG complex formation and/or binding at the *Ink4a* locus drives this effect. We also suspect that manipulating TGFβ signaling can reprogram the *Ink4a* locus to modify $p16^{INK4a}$ expression and affect cell cycling (**Figure 2**). On a mechanistic level, inhibiting TGFβ signaling has the potential to destabilize Smad-containing TrxG complexes at the *Ink4a* locus by interfering with Smad2/3:coSmad4 complex migration to the nucleus, thereby decreasing nuclear Smad protein availability.

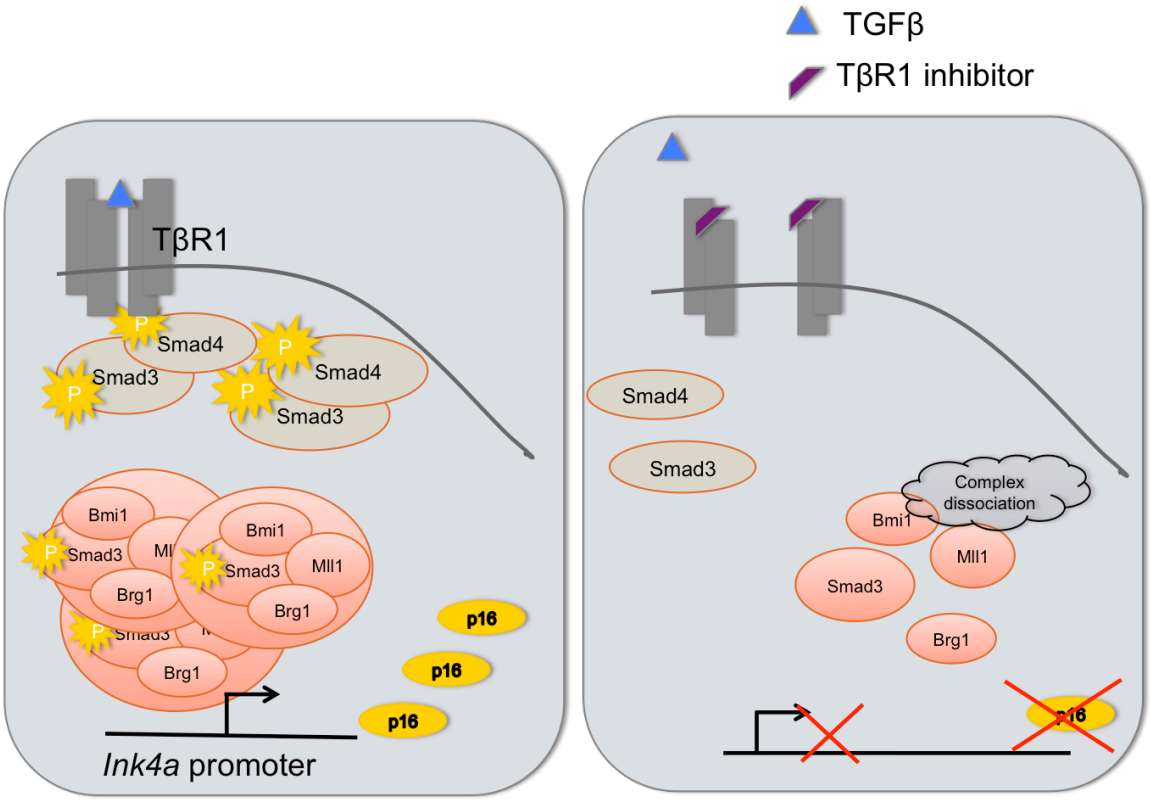

Figure 2: Schematic depicting the rationale for using TβRI inhibitors to stimulate aged β-cell proliferation.

Pancreatic β-cell proliferation decreases dramatically shortly after birth and then regresses more slowly in adults [24-27]. Based on these findings, islets are thought to lose their capacity for replication with age. A major shortcoming of the research on Pdx-1 and Nkx6.1 so far is that robust proliferative effects have only been demonstrated in young rodent islets, and these effects are strongly attenuated in older rodent islets and human islets, the latter often coming from middle aged to elderly cadaveric donors. Identification of methods to enable or potentiate the effects of these promising

transcription factors in humans is therefore a key goal of our laboratory. The work described here is an effort to determine if TGFβ inhibitors are capable of stimulating aged β-cell proliferation, and whether these inhibitors have a synergistic effect on aged β-cell proliferation when combined with Nkx6.1 or Pdx-1 overexpression.

2. Results

Nkx6.1 overexpression was achieved by treating isolated rat islets with an adenoviral construct containing the CMV promoter driving expression of the hamster Nkx6.1 cDNA (AdCMV-Nkx6.1), mouse Pdx-1 cDNA (AdCMV-Pdx-1), or a control virus containing GFP cDNA (AdCMV-GFP) [41,42]. All experiments were conducted in islets isolated from male Wistar rats. Islets were either isolated from young rats (8-12 weeks of age) or from retired male breeders (8-10 months of age) for experiments with aged islets.

2.1 Nkx6.1 and Pdx-1 Overexpression in Aged Islets

Studies on the effects of Nkx6.1 or Pdx-1 overexpression on β-cell proliferation have focused on islets from young rats [19,43]. Here we sought to compare proliferative responses in islets from young versus older animals. Following isolation, islets were allowed to recover overnight, were treated with AdCMV-Nkx6.1, AdCMV-Pdx-1, or AdCMV-GFP adenoviruses, and were cultured for an additional 72 hours after viral treatment. Proliferation was detected by adding ^{3}H-thymidine to the culture medium for the last 24 hours prior to islet harvest. As shown in **Figure 3**, Nkx6.1 or Pdx-1 overexpression produced the previously observed increases in ^{3}H-thymidine incorporation in islets from young rats (2 months of age), but had only a trend to increase proliferation in islets isolated from aged rats (approximately 8 months of age). Presumably, the impaired proliferative response of aged islets indicates that critical

factors that drive β-cell regeneration are no longer present or become inhibited as time progresses.

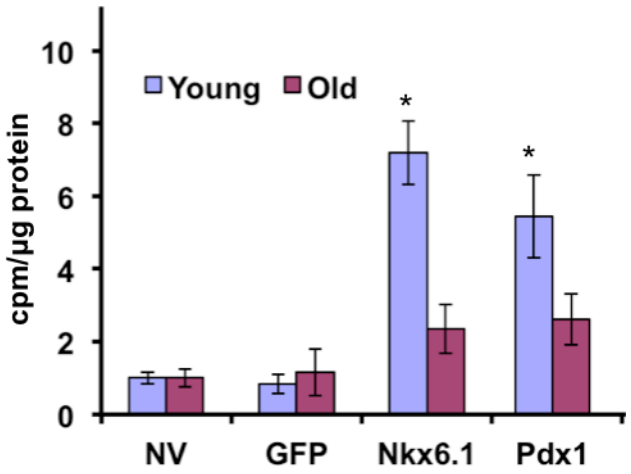

Figure 3: Proliferative effect of Nkx6.1 and Pdx-1 overexpression in cultured islets from 2 and 8 month old rats. Rat islets were treated with adenovirus for 18h and then cultured for an additional 80h, at which time uptake of ^{3}H-thymidine into genomic DNA was measured. *, *p* value < 0.05 compared to Ad-GFP and untreated islets. Data represent the means ± standard errors from four independent experiments, each performed in triplicate. Experiments and analysis done by Jeff Tessem, PhD.

Due to the limiting effect of $p16^{INK4a}$ on rodent β-cell proliferation in models of regeneration and proliferation with age, we attempted to perform RNAi-mediated knockdown of p16INK4a to determine if it was responsible for preventing replication in response to Nkx6.1 or Pdx-1 overexpression in rat aging. Eight unique RNAi sequences were designed to target the rat $p16^{INK4a}$ transcript and adenoviruses were generated to

achieve their expression in islets using an adenoviral backbone containing CMV-GFP (Adsi.p16/GFP). Quantitative PCR analysis utilized three assays targeting the cdkn2a locus, two that target exon 1α to specifically amplify the $p16^{INK4a}$ transcript and one that targets the exon 2-3 boundaries to amplify either $p16^{INK4a}$ or $p19^{ARF}$ transcripts. $p16^{INK4a}$ transcript was measured in young and old primary rat islets and was at times below the level of detection. Therefore, mRNA levels were quantified for three additional genes of different abundances (Cyclin E, Nkx6.1, and Pdx-1) for comparison and all genes were normalized to cyclophilin B. Nkx6.1 and Pdx-1 were most abundant, followed by cyclin E, p19, and p16 (**Figure 4A**). There is roughly 100 times the amount of cyclophilin B transcript as there is p19. Both $p16^{INK4a}$ assays revealed ~1000 times the amount of cyclophilin B transcript as there is p16, and closer to 10,000 times as much in aged islets. We expected to see higher $p16^{INK4a}$ mRNA levels in aged islets, given the reports of $p16^{INK4a}$ mRNA and protein accumulation in mouse islets with age [28,29]. Curiously, we observed the opposite as shown in **Figure 4B**, with islets isolated from 8-10 month old rats showing a decline in mRNA levels of p16 but not p19 relative to 2-month islets. Islets treated with Adsi.p16/GFP were GFP-positive and also expressed the adenoviral envelope gene L4 to a similar level as Ad-GFP control virus, suggesting that the viruses were infectious. However, the low abundance of the rat $p16^{INK4a}$ transcript made it difficult to detect any change in expression induced with any of the eight p16 siRNA adenoviruses, and immunblot analysis was also unreliable, so we were unable to

reliably demonstrate p16 knockdown (**Figure 4**). This problem was not due to the p16 antibody used, as a strong band was observed when 3T3-L1 cells were used as positive control (**Figure 4C**).

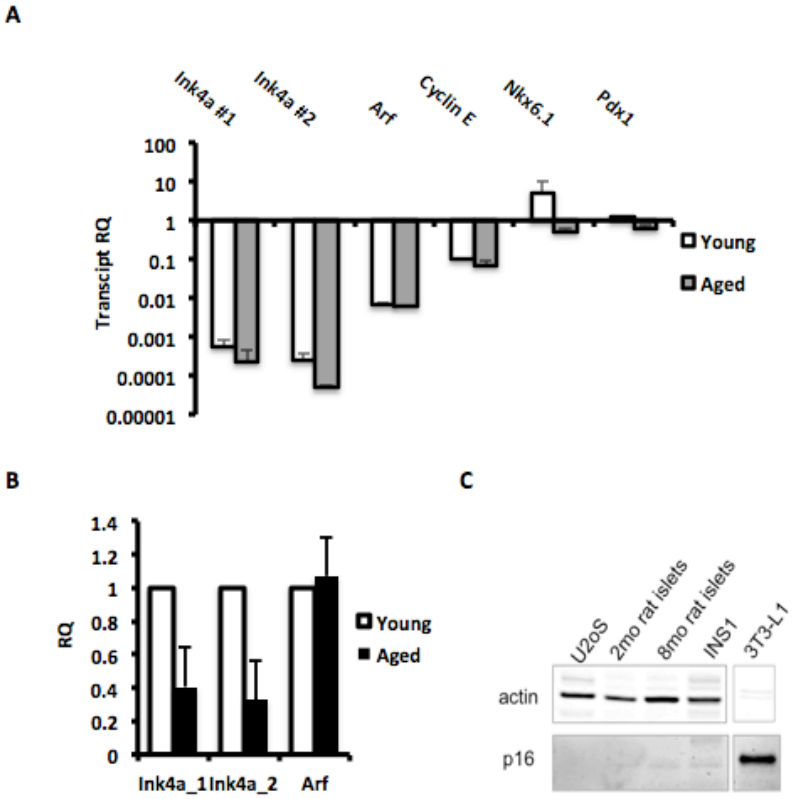

Figure 4: p16^{INK4a} expression is low in cultured islets from 2 and 8 month old rats. Islets were cultured for 24-48h before harvesting for mRNA or protein expression analysis. (A) qPCR was used to measure mRNA levels in young and old rat islets. RQ is relative to cyclophilin B and values are expressed on a log scale. Two independent qPCR assays were used for p16 (exon 1α) to confirm results. (B) qPCR analysis of rat cdkn2a transcripts relative to 2-month old untreated islets. n=6 young, n=3 old. (C) Representative western blot of 2 and 8-month old islets, rat 832/13 insulinoma cell line lysate, a negative control lysate (U20S), and a positive control (3T3-L1 cell) lysate.

2.2 TβRI Inhibition in Young Islets

Given the very low levels of $p16^{INK4a}$ transcript in rat islets and our technical difficulties with demonstration of siRNA-mediated suppression of p16 expression, we embarked on an alternative approach of using TGFβ receptor I (TβRI) inhibitors to increase aged rat β-cell proliferation via dissolution of the Mll1 complex at the *Ink4a* locus (**Figure 2**). We used two different inhibitors of TGFβ signaling, SB525334 (IC_{50} – 14.3nm) and LY2157299 (IC_{50} 56nM) [44,45]. Both are potent inhibitors of TβRI (ALK5) that block TβRI phosphorylation, preventing nuclear localization of Smad2/3. Not only is SB more potent than LY for TβRI inhibition, it also uniquely inhibits the activin receptor ALK4, with approximately one-fourth the potency compared to its effect on TβRI (ALK4 IC_{50} 58nM) [44].

Initially we determined whether these inhibitors had any direct proliferative effect in young rat islets in vitro [47-51]. Islets from 2-month old rats were cultured with 10μM SB or LY and proliferation was measured using a ^{3}H-thymidine incorporation assay. In these experiments, islets were cultured with ^{3}H-thymidine for the entire 72h of exposure to TGFβ inhibitors to extend the time period for detecting proliferating cells. Both inhibitors caused an increase in thymidine incorporation compared to DMSO-treated or untreated controls (**Figure 5**). The increase in proliferation is supportive of the hypothesis that inhibiting TGFβ signaling has a positive effect on cell cycle progression.

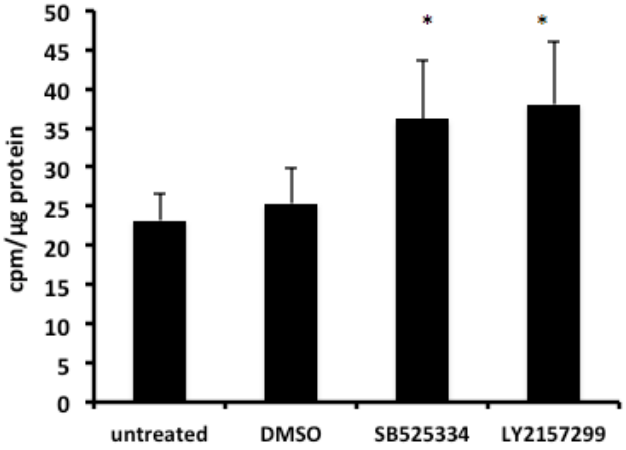

Figure 5: TβRI inhibition stimulates ^{3}H-thymidine incorporation in 2-month old rat islets. Rat islets were cultured with TβRI inhibitors SB525334 (10μM), LY2157299 (10μM) or 0.1% DMSO. Inhibitors and 3H-thymidine analogue were added each 24h. Uptake of ^{3}H-thymidine into genomic DNA was measured after 72h. *, p value < 0.001 compared to DMSO and untreated. Data represent the means ± standard errors from four independent experiments, each performed in triplicate.

2.3 TβRI Inhibition Combined With Pdx-1 or Nkx6.1 Overexpression in Young Islets

Based on the increase in ^{3}H-thymidine incorporation with TGFβ inhibitors (**Figure 5**), we postulated that disrupting TGFβ signaling might potentiate Nkx6.1- or Pdx-1-driven islet cell replication. To test this possibility, islets were treated with AdCMV-Nkx6.1, AdCMV-Pdx-1, or AdCMV-GFP adenovirus for 18 hours and then cultured for an additional 72 hours with 10μM SB525334 or LY2157299 TβRI inhibitor and ^{3}H-thymidine added daily. Pdx-1 overexpression alone caused a 2.3 fold increase in thymidine incorporation in comparison to AdCMV-GFP controls (**Figure 6A**). SB525334 completely blocked the effect of Pdx-1 overexpression (Ad-Pdx1 with DMSO 35.3 ± 2.45

cpm/μg, Ad-Pdx-1 with SB 15.8 ± 4.2 cpm/μg $p < 0.0001$) and diminished incorporation levels to that of AdCMV-GFP treated islets. Treatment with LY2157299 also impaired Pdx-1-induced proliferation as measured by ^{3}H-thymidine incorporation, but to a lesser extent (Ad-Pdx-1 with DMSO 35.3 ± 2.45 cpm/μg, Ad-Pdx-1 with LY 24.7 ± 1.50 cpm/μg, $p < 0.0001$).

Young islets treated with AdCMV-Nkx6.1 without TβRI inhibitors showed a robust increase in ^{3}H-thymidine incorporation that was approximately 4.7 times that of AdCMV-GFP treated islets (**Figure 6B**). Islets co-cultured with SB TβRI inhibitor had a 37% reduction in Nkx6.1-induced islet replication (Nkx6.1 with DMSO 108 ± 7.28 cpm/μg, Nkx6.1 with SB 68.1 ± 17.1 cpm/μg, $p < 0.0001$). Treatment with LY did not affect Nkx6.1-induced proliferation as measured by ^{3}H-thymidine incorporation (Ad-Nkx6.1 with DMSO 108 ± 7.28 cpm/μg, Ad-Nkx6.1 with LY 101 ± 7.98 cpm/μg, ns).

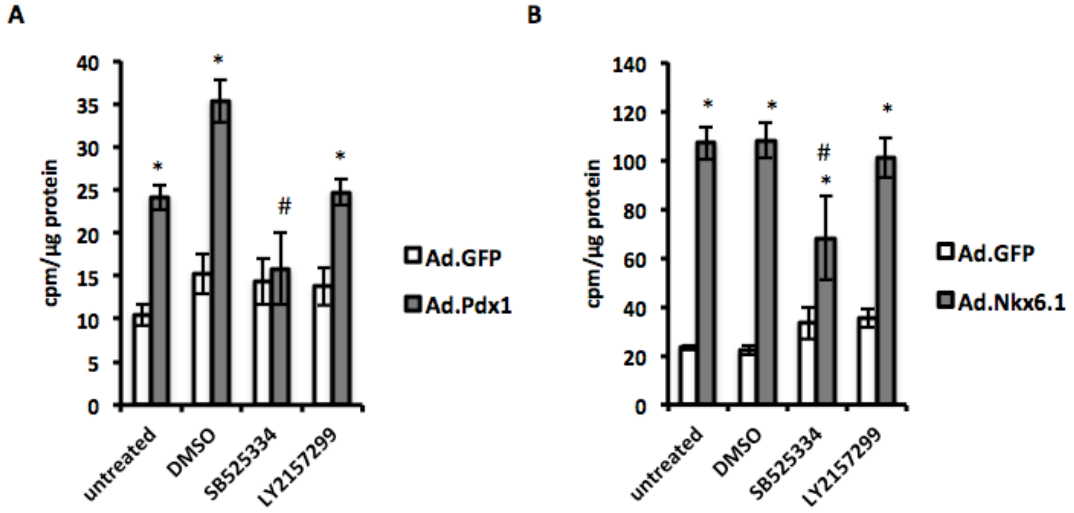

Figure 6: SB525334 blocks Pdx1 and Nkx6.1-stimulated ^{3}H-thymidine incorporation in 2-month old rat islets. Rat islets were treated with AdCMV-Pdx-1, AdCMV-Nkx6.1, or AdCMV-GFP and cultured with TβRI inhibitors SB525334 (10μM), LY2157299 (10μM) or 0.1% DMSO. Inhibitors and ^{3}H-thymidine analogue were added daily. Uptake of ^{3}H-thymidine into genomic DNA was measured after 72h. *, a p value < 0.001 compared with Ad-CMV-GFP islets receiving the same drug. #, a p value < 0.001 compared with other treatment groups transduced with (A) Ad-CMV-Pdx-1 or (B) Ad-CMV-Nkx6.1. Data represent the means ± standard errors from one experiment, performed in triplicate.

2.4 TβRI Inhibition in Aged Islets

Prior studies show that, by 8 months of age, rodent islets lose their capacity for β-cell replication to compensate for increased metabolic demands [46]. To investigate whether TGFβ inhibitors were capable of stimulating aged β-cell proliferation, islets were isolated from 8-10 month old rats and treated with AdCMV-Nkx6.1, AdCMV-Pdx-1, or AdCMV-GFP adenovirus for 18 hours and then cultured for an additional 72 hours

with 10μM SB525334 or LY2157299 TβRI inhibitor and ^{3}H-thymidine added daily. As shown in **Figure 7**, SB treatment caused a trend to increase ^{3}H-thymidine uptake that was not statistically significant (DMSO 35.0 ± 6.23 cpm/ug; SB 66.1 ± 22.1 cpm/μg, p=0.16). Notably, LY2157299 caused a significant increase in ^{3}H-thymidine incorporation (DMSO 35.0 ± 6.23 cpm/μg; LY 95.4 ± 28.9 cpm/μg, $p < 0.004$).

Because islets from aged rodents are resistant to Nkx6.1 or Pdx-1-induced replication we asked whether inhibition of TGFβ signaling could unveil an effect of these factors to activate aged islet cell replication. Nkx6.1 overexpression caused a two-fold increase in thymidine incorporation that did not achieve statistical significance (p=0.114), whereas Pdx-1 overexpression had no proliferative effect on aged islets in vitro (**Figure 7**).

Islet cell proliferation did not increase significantly by combining SB with Pdx-1 overexpression (SB with Ad-Pdx-1 70.8 ± 24.7 cpm/μg, SB with Ad-GFP 66.1 ± 22.1 cpm/μg, DMSO with Ad-GFP 35 ± 6.23 cpm/μg, *ns*). Islet cell proliferation increased significantly when SB was combined with Nkx6.1 overexpression compared with Ad-GFP, DMSO treated islets (SB with Ad-Nkx6.1 86.9 ± 2.49 cpm/μg, SB with Ad-GFP 66.1 ± 22.1 cpm/μg, DMSO with Ad-GFP 35.0 ± 6.23 cpm/μg, $p < 0.026$ in comparison to DMSO with Ad-GFP). No significant increase was observed in islets treated with SB + Nkx6.1 compared to either treatment alone.

Islet cell proliferation did not increase significantly by combining LY with Pdx-1 overexpression (LY with Ad-Pdx1 72.0 ± 14.8 cpm/μg, LY with Ad-GFP 95.4 ± 28.9 cpm/μg, DMSO with Ad-GFP 35.0 ± 6.23 cpm/μg, *ns*). Islet cell proliferation increased significantly when LY was combined with Nkx6.1 overexpression compared with Ad-GFP, DMSO treated islets (LY with Ad-Nkx6.1 106 ± 12.9 cpm/μg, LY with Ad-GFP 95.4 ± 28.9 cpm/μg, DMSO with Ad-GFP 35.0 ± 6.23 cpm/μg, $p < 0.003$ in comparison to DMSO with Ad-GFP). No significant increase was observed in islets treated with LY + Nkx6.1 in comparison to either Ad-Nkx6.1 or LY treatment alone.

Pdx-1 and Nkx6.1 overexpression alone did not significantly affect thymidine incorporation in comparison to AdCMV-GFP-treated islets. When Pdx-1 overexpression is combined with TGFβ inhibitors, no additive effect was observed (**Figure 7**). The most effective combination in these experiments was Nkx6.1 + LY, which combined caused a 3-fold induction in ^{3}H-thymidine incorporation in aged rat islets. Neither inhibitor enhanced the efficacy of Pdx-1 for proliferation.

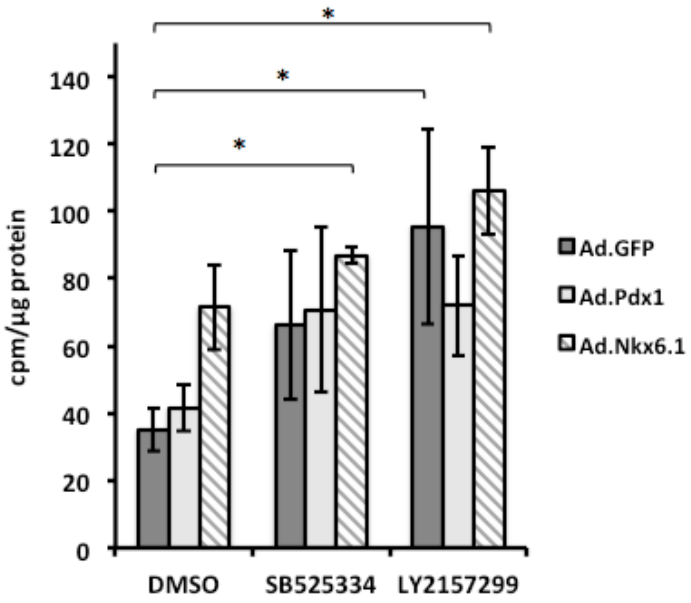

Figure 7: TβRI inhibition stimulates ^{3}H-thymidine incorporation in aged rat islets. Aged islet cell proliferation treated with TGFβ inhibitors in the context of Nkx6.1 or Pdx-1 overexpression assessed by 3H-thymidine incorporation. Rat islets were treated with AdCMV-Pdx1, AdCMV-Nkx6.1, or AdCMV-GFP and cultured with TβRI inhibitors SB525334 (10μM), LY2157299 (10μM) or 0.1% DMSO. Inhibitors and ^{3}H-thymidine analogue were added daily. Uptake of ^{3}H-thymidine into genomic DNA was measured after 72h. *, a p value < 0.05 in comparison with DMSO/AdCMV-GFP treated islets. Data represent the means ± standard errors from three independent experiments, each performed in triplicate.

2.5 EdU/Insulin Immunofluorescence in Aged Islets

Rat islets in culture are not purely β-cells, but are also comprised of fibroblasts, exocrine cells, and other endocrine cell types. Since ^{3}H-thymidine incorporation assays do not distinguish between cell types, we cannot make any conclusions from them about whether β-cell replication is specifically affected by inhibiting TGFβ signaling. We therefore performed a set of experiments with the nucleotide analogue EdU, which is

incorporated into newly synthesized DNA and can be imaged with fluorescence microscopy. When combined with insulin staining to identify β-cells, this method allows quantification of β-cells that synthesized DNA over the course of the experiment. To investigate whether TGFβ inhibitors were capable of specifically stimulating β-cell proliferation, islets were treated with AdCMV-Nkx6.1, AdCMV-Pdx-1, or AdCMV-GFP adenovirus for 18 hours and then cultured for an additional 72 hours with 10μM SB525334 or LY2157299 TβRI inhibitor and EdU added daily. Islets were dispersed into single cells just before fixation to enhance immunofluorescence detection, visualization, and quantification.

In 2-month old islets, TGFβ inhibition had a mild positive effect on the total number of islet cells that were EdU positive (**Figure 8A**), but did not alter the number of EdU positive β-cells (**Figure 8B**). TβRI inhibition by SB increased the percentage of EdU positive islet cells approximately 2 fold, whereas LY had a smaller effect on islet cell EdU incorporation (AdCMV-DMSO 1.6%, LY 2.3%, SB 3.3%). As shown in **Figure 8B**, quantification of the percentage of β-cells that were both EdU and insulin positive revealed that TGFβ inhibitors diminished the number of β-cells that had undergone replication compared to that of DMSO-treated islets (SB 0.21%, LY 0.11%, DMSO 0.28%). As anticipated, Pdx-1 or Nkx6.1 overexpression resulted in a respective 3- and 7-fold increase in the number of β-cells undergoing cell division in comparison to AdGFP-treated islets (**Figure 8B**), which correlates with the increase in ^{3}H-thymidine

incorporation shown in **Figure 6**. These results demonstrate that in young islets, Nkx6.1 or Pdx-1 overexpression increases the number of β-cells undergoing cell division, but TGFβ inhibition does not, even when combined with overexpression of the transcription factors.

To investigate whether TGFβ inhibitors are capable of stimulating aged β-cell proliferation, we conducted experiments in islets isolated from 8-10 month old rats. We found that LY increased the total number of EdU positive cells compared to DMSO-treated islets (DMSO 8.01+2.15%, LY 13.8± 1.66%, p <0.01). Combining Pdx-1 overexpression with LY had an additive effect on islet cell proliferation, but did not affect replicating β-cells (DMSO/AdCMV-Pdx1 10.1±2.84%, LY/GFP 13.8 ± 1.66%, LY/Pdx1 22.7%, p <0.03). Neither TGFβ inhibitor increased the number of β-cells undergoing replication, either in the presence or absence of overexpression of the transcription factors. Our initial observation that aged islets have a failed proliferative response to Nkx6.1 or Pdx-1 overexpression (**Figure 7**) was refortified here by a failure to increase EdU positive cells. These results demonstrate that TβRI inhibition is not effective in stimulating aged β-cells to undergo replication. Based on these findings, we conclude that inhibition of TβRI is not a method for enabling Nkx6.1 or Pdx-1-mediated β-cell replication, especially in aged, insulin-positive cells. Perhaps its therapeutic benefit lies in targeted cancer therapies, since it appears to have disparate effects based on age and cell type.

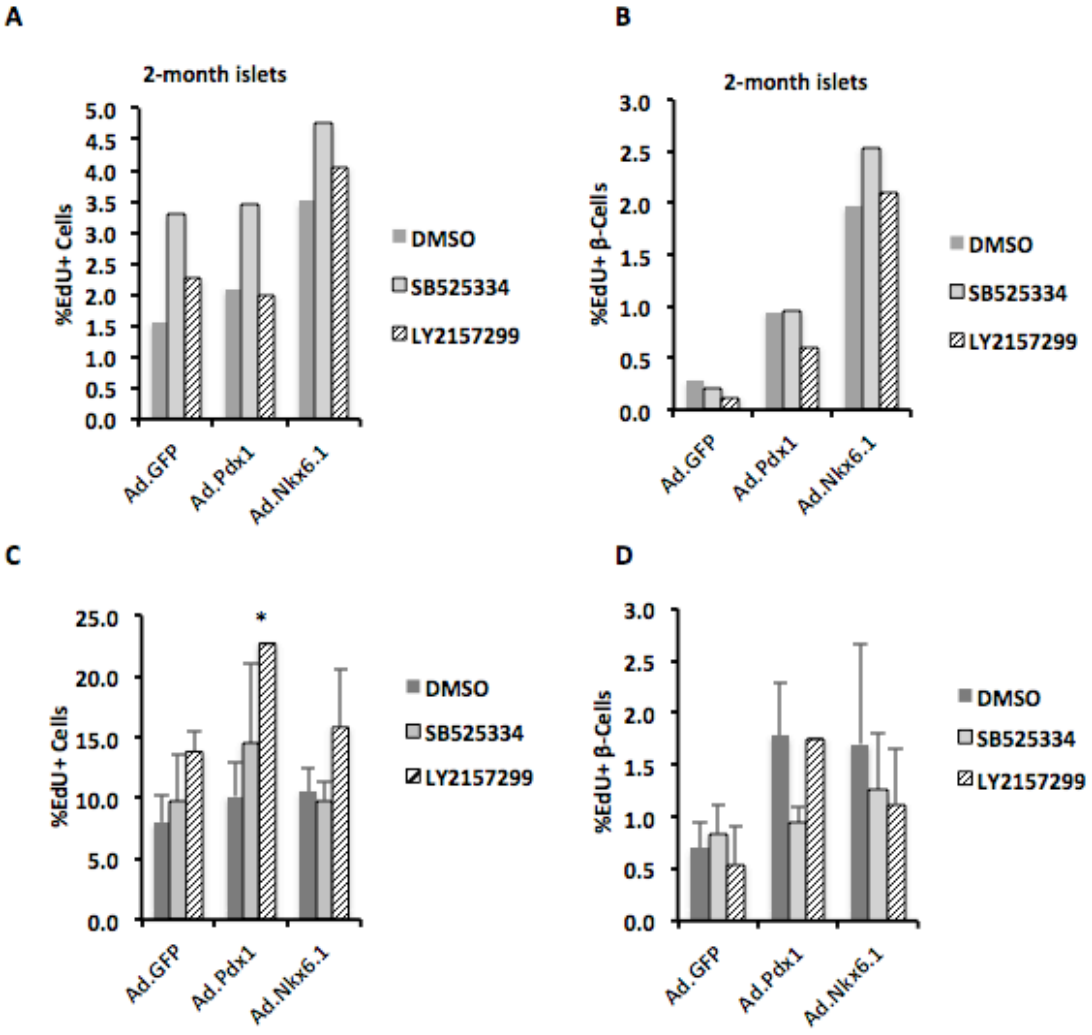

Figure 8: Percentage of EdU and insulin positive cells. Islets were isolated from 2 month old rats (A, B) or 8-10-month old rats (C, D), infected with adenovirus for 18h, then cultured with TβRI inhibitors SB525334 (10μM), LY2157299 (10μM) or 0.1% DMSO with EdU added daily. 72h after adding inhibitors, islets were dispersed, fixed, and stained for insulin, EdU, and Dapi. (A) Percentage of total cells that were EdU-positive from 2-month old rats. (B) Percentage of β-cells that were EdU positive from 2-month old rats. (C) Percentage of total cells that were EdU-positive from older islets. (D) Percentage of β-cells that were EdU positive in older islets. *, p value < 0.02 in comparison to all other conditions. Data represent 3-5000 cells per condition from one (A, B) and two (C, D) independent experiments.

3. Discussion

The proliferative effects of Pdx-1 and Nkx6.1 on β-cell replication are strongly attenuated in older rodent islets and human islets. Finding a means to unveil the proliferative effects of Pdx-1 and Nkx6.1 transcription factors in human islet cells is a key goal. Evidence that the age-dependent decline in β-cell replication is a consequence of cellular $p16^{INK4a}$ accumulation suggests that suppressing $p16^{INK4a}$ protein could be a prerequisite for Nkx6.1 or Pdx-1-induced aged β-cell proliferation. We abandoned our initial strategy of RNAi-mediated silencing of $p16^{INK4a}$ transcript due to the low abundance of the rat $p16^{INK4a}$ transcript in islet cells, which made it difficult to detect any siRNA-mediated changes in expression. We instead chose to study the effect of TGFβ inhibitors, in light of recent reports implicating TGFβ signaling in the regulation of the *Ink4a* locus [47]. In one report, activation of TGFβ signaling in mouse embryonic fibroblasts caused an increase in $p16^{INK4a}$ expression and cell phenotype that mimicked replicative senescence [47]. In the current study, we studied the effects of two TβRI inhibitors on islets from young and aged rats, with a goal of testing if these compounds are capable of enhancing or enabling Nkx6.1 or Pdx-1-stimulated β-cell proliferation.

In 2-month old islets, the two TβRI inhibitors we tested had a mild positive effect on the number of total islet cells that were EdU positive (**Figure 8A**), but did not affect replication of the β-cell population (**Figure 8B**). As anticipated, Pdx-1 or Nkx6.1 overexpression produced a respective 3- and 7-fold increase in the number of replicating

β-cells in comparison to AdGFP-treated islets (**Figure 8B**), which is well correlated with the boost in ^{3}H-thymidine incorporation they induced in **Figure 6**. These results demonstrate that in young islets, TGFβ inhibitors do not increase the number of β-cells undergoing cell division, even when combined with Nkx6.1 or Pdx-1 overexpression.

In aged islets, two distinct methods for measuring cell proliferation revealed that the TβRI inhibitor LY stimulates total cell proliferation, but does not change β-cell proliferation. We have therefore identified a compound that is capable of inducing a modest, ~2-fold increase in aged islet cell proliferation, but that does not stimulate replication of the β-cell population as we intended. Taken together, we identify LY2157299 as a compound having a modest effect on non-β-cells within the aged rat islet cell pool.

Our laboratory has observed that co-overexpression of Pdx-1 and Nkx6.1 has an additive effect on islet proliferation, suggesting that they regulate discrete sets of genes to stimulate β-cell proliferation. Consistent with this evidence for discrete proliferative pathways is our recent discovery that Pdx-1 stimulates proliferation of both α- and β-cells, and that the increased islet cell proliferation occurs in cells that do not overexpress Pdx-1, implicating a cell extrinsic soluble factor [43]. Moreover, these studies have uncovered a novel interaction between Pdx-1 and TGFβ signaling in which Pdx-1, but not Nkx6.1, induces the expression of a gene encoding a ligand in the TGFβ superfamily that stimulates both rat and human islet cell proliferation. Microarray and GO analysis

identified Inhbb, a gene encoding subunits of the TGFβ superfamily ligand activin, as a Pdx-1 responsive gene that potentiates Pd-1 stimulated rat islet proliferation when both genes are overexpressed. Moreover, activin receptors RIIA and RIIB were required for the full proliferative effect of Pdx-1. These studies highlight the involvement of non-cell autonomous TGFβ signaling activation during Pdx1-stimulated islet cell proliferation by Pdx-1 target genes.

Consistent with the recent findings about mechanisms of Pdx-1 signaling in islets, the current study shows that ALK5 and activin receptor ALK4 inhibitor SB completely blocked Pdx-1 stimulated ^{3}H-thymidine incorporation in 2-month old islets, whereas the ALK5-specific inhibitor LY partially impaired Pdx-1 stimulated ^{3}H-thymidine incorporation. These findings are supportive of our recent report that activin receptors RIIA and RIIB are necessary for the full proliferative effect of Pdx-1. Curiously, in young islets, we did not observe this impairment in Pdx-1 stimulated proliferation when we quantified total cell proliferation by EdU incorporation, and in fact, SB augmented Pdx-1 stimulated proliferation using this method (**Figure 8**). Also, in terms of β-cell proliferation, LY, but not SB reduced the Pdx-1 effect. In islets from old rats, SB, but not LY, prevented the modest Pdx-1 induced increase in β-cell proliferation. In general, the findings of the current study support the idea that Pdx-1 induced β-cell proliferation via a TβRI-mediated mechanism since in most experiments, SB inhibited the effects of Pdx-1 overexpression.

We observed partial impairment of Nkx6.1-stimulated islet cell proliferation with SB treatment in young islets. In aged islets, both TGFβ inhibitors improved Nkx6.1-stimulated ^{3}H-thymidine incorporation. Addition of LY to AdNkx6.1-treated islets caused a further boost in the overall number of replicating cells in comparison to AdNkx6.1-treated islets. We observed a similar effect when we assessed proliferation with ^{3}H-thymidine incorporation. However, none of the conditions caused an increase in the number of replicating β-cells. Thus, inhibition of TGFβ-signaling does not improve Nkx6.1-stimulated β-cell proliferation in older islets.

At the onset of this study, we anticipated that disruption of TGFβ signaling would have an additive effect on islet cell proliferation in the context of Pdx-1 or Nkx6.1 overexpression. We postulated that TGFβR inhibitors could facilitate aged β-cell proliferation by dissolution of the TrxG-Mll1 protein complex that has been shown to control activation of *Ink4a* expression in aging β-cells. Experiments in MEFs demonstrate that re-expression of Ezh2 was insufficient to repress Ink4a unless combined with loss of Mll1, similar to the mechanism occurring in β-cells (**Figure 2**). Therefore, TrxG-PcG mechanisms governing *Ink4a* expression and replication capacity may be a conserved mechanism across multiple cell types and tissues. Here we provide evidence that TβRI inhibitors cause a modest increase in aged islet cell proliferation, but do not specifically affect proliferation of the β-cell population. Further studies are necessary to determine the identity of the cells that replicate in response to TGFβ signaling inhibition.

4. Methods

4.1 Recombinant Adenoviruses for Gene Overexpression

For gene overexpression studies, cytomegalovirus promoter-driven recombinant adenoviruses containing the hamster Nkx6.1 cDNA (AdCMV-Nkx6.1), green fluorescent protein (GFP) cDNA (AdCMV-GFP), and mouse Pdx1 cDNA (AdCMV-Pdx-1) were prepared using the pJM17 adenoviral vector as previously described (41,42). All recombinant adenoviruses were screened for E1A deficiency using a quantitative-PCR assay.

4.2 Islet Culture

Pancreatic islets were harvested and cultured as previously described (18,19) from male Wistar rats under a protocol approved by the Duke University Institutional Animal Care and Use Committee. Pools of 200 islets per condition were cultured in 2ml complete medium and treated with viruses at a concentration of 5×10^9 particles/ml medium. After 18h exposure to adenovirus, islets were moved to fresh virus-free media, and islets were cultured for various times after treatment as indicated in the figure legends, with fresh medium daily. Rat islets were treated with TGFβ inhibitors SB525334 and LY2157299 (Selleck Chemicals) at 10uM for 72h. Fresh media, inhibitor, and EdU or 3H-thymidine were added each day. Experiments were conducted with intact islets and only dispersed immediately prior to fixation for ease of immunofluorescence analysis.

4.3 ^{3}H-Thymidine Assay

^{3}H-thymidine (Perkin Elmer) was added to fresh media at a final concentration of 1 μCi/ml to pools of ~100 islets every 24h of cell culture unless otherwise noted. Groups of 30 islets were picked in triplicate, washed, and centrifuged twice at 300 × *g* for 3 min at 4°C. DNA was precipitated with 600μl of cold 10% trichloroacetic acid and solubilized by the addition of 80μl of 0.3 N NaOH. The amount of [^{3}H]thymidine incorporated into DNA was measured by liquid scintillation counting and normalized to the amount of total cellular protein, as determined using Pierce Micro BCA Kit (Thermo Scientific).

4.4 Islet Dispersion

Pools of 50 islets per condition were washed in PBS (without Ca2+/Mg2), resuspended in 0.05% Trypsin/EDTA (Sigma) and incubated at 37°C for 15 minutes with pipet agitation every 5 minutes until dispersal was complete. Islets were then washed with RPMI + 10% FBS, resuspended in 10ul PBS, and plated on poly-d-lysine coated coverslips (BD Biosciences). After 30 minutes in a 37°C incubator with 5% CO_2, cells were fixed in 4% formaldehyde for 15 minutes.

4.5 EdU Immunofluorescence

For EdU labeling, a 1:1000 dilution of EdU labeling reagent (Invitrogen) was added to islet culture medium for the last 18h of cell culture unless otherwise specified. Islets were dispersed using trypsin-EDTA, plated on poly-D-lysine-coated-coverslips (BD), and fixed in 4% formaldehyde. EdU positive cells were stained using the Click-iT

EdU 555 kit (Invitrogen) according to the manufacturer's protocol. Insulin staining was performed in tandem, using guinea pig anti-insulin antibody (1:200; Invitrogen) followed by detection with Alexa Fluor 488 goat anti-guinea pig IgG or Alexa Fluor 647 goat anti-guinea pig IgG (1:2,000; Invitrogen). Cells were counterstained with DAPI (Fluorsave). All images were acquired with a 10x objective using an Axioplan2 microscope (Zeiss) and QImaging Retiga EXi camera and Openlab software. Cells were quantified using ImageJ.

4.6 Quantitative PCR

RNA was isolated using the RNeasy Micro Kit (Qiagen), and cDNA was made with iScript (Bio-Rad. Quantitative PCR assays were performed on the ViiA7 detection system and software (Applied Biosystems). The primers for Cyclin E, cdkn2a (exon1α), cdkn2a (exon 2-3), Nkx6.1, and Pdx1 were Taqman Assays (Applied Biosystems). Custom p16 assay sequences are as follows: Forward 5' ACC AAA CGC CCC GAA CA, Reverse: 5' GAG AGC TGC CAC TTT GAC GT, Probe 5' TCG GTC GTA CCC CGA TAC AGG TGA [29]. All rat islet mRNA levels were normalized to endogenous PPIB levels (IDT; Assay Rn.PT.39a.22214850).

4.7 Immunoblot Analysis

Cells or islets were harvest and lysed in ice-cold RIPA buffer (Sigma) with protease inhibitors (Pierce). Following centrifugation at 13,000rpm for 15min at 4°C, protein concentration was determined with Pierce Micro BCA Assay (Thermo Scientific).

20-30ug per lane was resolved on a 4 to 12% NuPAGE gel (Invitrogen) then transferred to a low-fluorescence PVDF membrane (Bio-Rad). After blocking with fish gelatin (Sigma), membranes incubated overnight at 4°C with primary antibodies: 1:400 anti-p16 (Santa Cruz; sc-1661; discontinued) or 1:2000 anti-γ-tubulin (Sigma; T5326). Goat anti-mouse IRDye 800CW (Li-Cor; 1:5,000) and Alexa Fluor 680 goat anti-rabbit antibodies (1:2,000) were used for detection using the Odyssey CLx system (Li-Cor).

4.8 Statistical Analysis

Data is presented as means ± standard errors of the mean (SEM). Multiple comparisons were tested by two-way analysis of variance (ANOVA) using JMP 11. For comparison between 2 groups, the unpaired, two-tailed Student's t test was used. *P*-values < 0.05 were considered significant.

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