Inhibition of TGF-beta signaling promotes human pancreatic beta cell replication

Sangeeta Dhawan\textsuperscript{a}, Ercument Dirice\textsuperscript{b}, Rohit N. Kulkarni\textsuperscript{b}, and Anil Bhushan\textsuperscript{c,1}

\textsuperscript{a}Department of Medicine, Division of Endocrinology, UCLA, Los Angeles, CA 90095.

\textsuperscript{b} Islet Cell and Regenerative Biology, Joslin Diabetes Center, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02215.

\textsuperscript{c} Diabetes Center, University of California, San Francisco, San Francisco, CA 94143.

\textsuperscript{1}Author for correspondence: anil.bhushan@ucsf.edu

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Abstract

Diabetes is associated with loss of functional pancreatic beta cells and restoration of beta cells is a major goal for regenerative therapies. Endogenous regeneration of beta cells via beta cell replication has the potential to restore cellular mass, however pharmacologic agents that promote regeneration or expansion of endogenous beta cells have been elusive. The regenerative capacity of beta cells declines rapidly with age, due to accumulation of p16<sup>INK4a</sup>, resulting in limited capacity for adult endocrine pancreas regeneration. Here, we show that TGF beta signaling via Smad3 integrates with the trithorax complex to activate and maintain <i>Ink4a</i> expression to prevent beta cell replication. Importantly, inhibition of TGF-beta signaling can result in repression of the <i>Ink4a/Arf</i> locus resulting in increased beta cell replication in adult mice. Furthermore, small molecule inhibitors of the TGF-beta pathway promote beta cell replication in human islets transplanted into NOD-<i>scid</i> IL2Rgnull (NSG) mice. These data reveal a novel role for TGF beta signaling in the regulation of the <i>Ink4a/Arf</i> locus and highlight the potential of using small molecule inhibitors of TGF-beta signaling to promote human beta cell replication.
Introduction

Replication of beta cells is the primary mechanism for maintenance and expansion of beta cell mass in response to changing insulin demands (1-4), and failure of such adaptive expansion can result in diabetes (5; 6). Induction of p16\textsuperscript{INK4a} expression, a product of the Ink4a/Arf locus, is typical in adult tissues (7; 8), and contributes to reduced self-renewal across mammalian tissues, including beta cells (9-11). This reflects in the reduced ability of the endocrine pancreas for adaptive expansion and regeneration (12; 13). The Ink4a/Arf locus has also been linked with type 2 diabetes in genome wide association studies (GWAS) (14). Thus, manipulation of mechanisms that regulate the Ink4a/Arf locus could serve a tool for promoting adult beta cell replication.

Several studies, including ours, have shown that distinct repressive complexes of polycomb group (PcG) proteins regulate beta cell replication during aging, by epigenetically targeting the Ink4a/Arf locus(15; 16). Polycomb repressive complex 2 (PRC2) contains histone H3K27 methyltransferase Ezh2 and marks the target chromatin with lysine 27 trimethylation of Histone H3 (H3K27me3). This directs the recruitment of Polycomb repressive complex 1 (PRC1 ; contains Bml1 and ubiquitin ligase-Ring1B), leading to the ubiquitination of lysine 119 in histone H2 (H2AK119)(11; 17). Together, these epigenetic modifications result in the repression of the Ink4a/Arf locus in juvenile beta cells to maintain replicative potential. The loss of polycomb complex binding in adult beta cells, results in the recruitment of histone methyltransferase Mll1, which mediates trimethylation of lysine 4 in histone H3 (H43K4me3), an activating histone modification (15; 16). In beta cells, Mll1 exists in a complex with histone
H3K27me3 demethylase JmjD3, which removes the repressive H3K27me3 modifications (18). Together, the recruitment of the Mll1-JmjD3 complex leads to the induction of p16<sup>INK4a</sup> expression (15; 16; 18), resulting in reduced beta cell replication. Our recent work shows that a combination of polycomb replenishment with loss of Mll1 complex binding can drive replication in adult beta cells (18).

While recent work has unraveled cellular signals such as PDGF that regulate the repression of p16<sup>INK4a</sup> expression and beta cell self-renewal in young adults (19), the signals that mediate the induction of Ink4a/Arf locus in late adult life are not known. Here, we report that TGF-beta signaling induces Ink4a expression leading to replicative decline in beta cells, through the recruitment of Smad3 as a part of the Mll1 complex. Smad3 is a downstream effector of the canonical TGF-beta signaling, and translocates to the nucleus upon its phosphorylation as a consequence of ligand binding to the TGFbeta receptor (20). We demonstrate that inhibition of TGF-beta signaling using small molecules can reduce the levels of p16<sup>INK4a</sup> in a conserved fashion across cell types. Finally, we show that small molecule inhibitors of TGF-beta signaling can be used to induce beta cell replication by lowering the cellular levels of p16<sup>INK4a</sup>. Together, these studies provide a therapeutically relevant example of how cellular signals modulate beta cell replication via targeting epigenetic pathways.

**Research Design and Methods**

**Animal maintenance**
For the experiments involving mouse islets and mouse in vivo regeneration studies, the animals were maintained by mating wildtype males and females on a C57BL/6J background. Male NOD Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NOD-scid IL-2Rg^{null}, NSG) (denoted NOD-scid-gamma or NSG) (8-10 weeks) mice were used for human islet transplant experiments. All animal experiments were performed in accordance with NIH policies on the use laboratory animals, and approved by the Animal Research Committee of the Office for the Protection of Research Subjects at UCLA, and Institutional Animal Care and Use Committee protocols at Joslin Diabetes Center.

Animal experiments

For experiments on the effect of TGF-beta inhibitor on mouse beta cell replication in vivo, wildtype C57BL6/J mice (age= 9 months) were divided into two groups (n=9 per group) and injected intraperitoneally with a single dose of either TGF-beta inhibitor SB431542 (5mg/Kg body weight), or vehicle control (50% (v/v) DMSO), ensuring that the volume injected was kept up to 100 microliter. The concentration of DMSO used as vehicle in these studies has previously been shown to have no adverse effects on mouse health in long-term studies (21). Pancreata from these mice were harvested and processed for histology and analyzed for proliferation after one week (4). For human islet graft experiments, male NSG mice of (n=4) were used for experiments and grafted with human islets (1000 islet equivalents, IEQ) under the kidney capsule as described previously (22; 23). One week post transplantation (when the islets developed vascular anastomosis), mice were injected intraperitoneally with vehicle or TGF-beta inhibitor (5mg/kg/BW, 50% (v/v) DMSO) twice a week for 3 weeks. Four weeks post-transplantation mice were intraperitoneally injected with BrdU (Sigma-Aldrich, St Louis,
MO) (100mg/kg/BW) for 3 days and sacrificed 6 hours after receiving the final BrdU injection. Blood was collected before, 2 and 4 weeks after islets were grafted for the measurement of human insulin (Human Insulin ELISA Kit, Mercodia) and human c-peptide (C-Peptide ELISA Kit, Mercodia). In vivo GSIS (3g/kg/BW, 20% dextrose) was performed at 3 weeks post-transplantation after O/N fasting. The graft-containing kidneys and pancreases were paraffin-embedded and prepared for histological analysis (24).

**Cell culture, islet isolation and culture, inhibitor treatments**

Mouse embryonic fibroblasts were maintained on dishes coated with 0.2% porcine gelatin, in DMEM based medium containing 10% FBS at 37 °C in 5% CO₂ environment. Mouse islets were isolated using the Liberase/DNase enzyme mix (Roche Diagnostics) as described (25). In brief, the pancreas was inflated with the enzyme mix through the common bile duct and digested at 37°C. Islets were enriched by gradient and hand picked. Human islet samples were obtained from healthy, adult, non-diabetic organ donors deceased due to acute traumatic or anoxic death and obtained from Integrated Islet Distribution Program (IIDP) as previously described (4). 100-200 islets were cultured in easy grip tissue culture dish (Falcon, 35mmX10mm) in Dulbecco’s Modified Eagle’s Medium (DMEM, Cellgro) containing 10% fetal bovine serum (FBS), supplemented with 11 mM D-glucose (mouse islets) or 5 mM D-glucose (human islets). For experiments involving the effect of TGF-beta on cellular aging and proliferation, early passage MEFs (P2) in culture were supplemented with 5.0 ng/mL TGF-beta (Peprotech), for two days (for proliferation assay) and for indicated number for days (for growth curve), with daily change of culture medium. For experiments with TGF-beta inhibitors, islets from old mice (8-12 months old) and late passage MEFs (P9-P12) were
cultured with one of the following TGF-beta inhibitors: SB431542 (15µM; Sigma-Aldrich); SB505124 (5µM; Sigma-Aldrich); or SD208 (10µM; Sigma-Aldrich), or control small molecule PI3K inhibitor LY294002 (10 µM; Sigma-Aldrich), for three days for replication analysis and indicated number of days for cell growth curves, with daily change of culture medium. For cell growth measurements, cell number was measured at indicated time points and following indicated treatments, using a hemocytometer and plotted as a function of time. Human islets from cadaveric donors were cultured with either SB431542 (40µM; Sigma-Aldrich); or vehicle control (DMSO), for 4 days and then harvested for replication analysis. Western blots with the indicated antibodies were performed as described previously(25).

**Immunofluorescence staining and morphometric analyses**

Standard immunofluorescence staining protocol was used to detect various protein markers in mouse pancreatic islet sections (4). Cultured islets were harvested, fixed in 4% paraformaldehyde, embedded in histogel matrix and processed according to the above protocol. MEFs were fixed as monolayers in culture (on gelatin coated plastic ware) for 20-30 minutes at room-temperature with the BD Cytofix/Cytoperm solution (BD Pharmingen) and washed thoroughly three times with 1X BD Perm-Wash buffer, followed by permeabilization at room temperature in the same buffer. Primary antibodies were diluted in the blocking solution (3% BSA in TBST) at the following dilutions: guinea pig anti-Insulin (diluted 1:400, Dako); mouse anti-Ki67 (diluted 1:50, BD Pharmingen); mouse anti-BrdU (1:2, GE Healthcare/Amersham RPN-202); anti-phospho histone H3 (pHH3, Millipore 1:200); rabbit anti-Pdx1 (dilated 1:200, Millipore); mouse anti-p16 (dilated 1:400; Santa Cruz sc-1661). Donkey- and goat-derived secondary antibodies conjugated to FITC or Cy3 were diluted 1:500 (Jackson
ImmunoResearch Laboratories). Slides were viewed using a Leica DM6000 microscope and images were acquired using Openlab software (Improvision). Proliferation index was calculated using Ki67, BrdU or pHH3 staining as a measure of cell proliferation, by co-staining the islet sections for Ki67 (or BrdU, or pHH3) and insulin or Pdx1, for beta cells and by co-staining for Ki67 with the nuclear marker DAPI, for MEFs. Beta-cell area was evaluated by point-counting morphometry on immunofluorescence-stained pancreatic sections. Cell death was detected by TUNEL assay (ApopTag S7100; Millipore). At least 2,000-3,000 beta cell nuclei were counted per animal.

**Immunoprecipitation and Western blotting**

2.5 mg. anti-Mll1 antibody (Millipore 05-765), or control IgG was used for each immunoprecipitation on MEF lysates, as previously published. Briefly, antibodies were bound to Protein G Sepharose (Millipore) for 2-3 hours and incubated with MEF extracts prepared in overnight at 4°C on a rotator. The immunoprecipitates were analyzed by Western blotting with antibodies against JmjD3 (Millipore 07-1434), and Smad3 antibody (Bethyl Laboratories A300-109A) and control Dnmt3a (Imgenex IMG-268A). For western blotting, samples were lysed in cell dissociation buffer (Invitrogen) supplemented with protease inhibitors cocktail (Calbiochem), as described. The following primary antibodies were used; mouse anti-p16 (Santa Cruz; sc-1661), mouse anti-Smad3 (BD Biosciences; 610842), rabbit anti-pSmad3 (07-1389), and mouse anti-beta tubulin (Sigma Aldrich; T8328). Each western blot is a representative from three independent experiments using different sample preparations.

**Chromatin Immunoprecipitation (ChIP) analysis**
Chromatin immunoprecipitation (ChIP) was performed as reported previously(26), with minor modifications. The islets (150-200 islets per group, made into single cell suspension) or MEFs (1X10^5 cells) were treated with 1% paraformaldehyde at room temperature for 20 minutes to crosslink the DNA with bound proteins. Formaldehyde was quenched by adding glycine to a final concentration of 125 mM, followed by cell lysis in 100 µL lysis buffer consisting of 50 mM Tris HCl pH 8.0, 10 mM EDTA, 1% SDS, supplemented with 1 x complete proteinase inhibitors (Calbiochem) and sonicated to yield DNA fragments with an average size of 500bp using Bioruptor (Diagenode). A total of 2.5 µg of antibody was bound to 20 µL Protein-A/G Dynabeads (Invitrogen), depending on the antibody isotype, for 2 hours at 4°C with agitation. The following antibodies were used: anti-3me-H3K27 (Millipore 07-449), anti-3me-H3K4 (Cell Signaling 9751S), anti JmjD3 (Millipore 07-1534), anti MLL1 (Millipore 05-765), anti-Smad3 (BD Biosciences; 610842), or normal mouse IgG as a control. One fourth fraction of the sonicated chromatin was incubated overnight at 4°C with the antibody-bead complexes in total volume of 200 µL in RIPA buffer containing 10 mM Tris HCl pH8.0, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS and 0.1% Na-deoxycholate supplemented with 1x complete proteinase inhibitors. After 4x washing with RIPA buffer and 2x with TE buffer, chromatin was eluted, followed by reverse-crosslinking at 68°C for 4 hours with vigorous agitation in the presence of Proteinase K (Sigma). The DNA fragments were then purified using phenol-chloroform extraction and ethanol precipitation. The resulting DNA was quantified and served as a template for the real-time PCR reactions, performed using ABI7900HT (Applied Biosystems), with 1X FastSybrGreen Mix (Applied Biosystems). The DNA enrichment after ChIP was estimated as percentage bound/input ratio, determined by
real-time PCR. The primers used to amplify the *Ink4a/Arf* locus have been described before (11; 16).

**Statistical methods**

All data were expressed as Mean±S.E. Mean and s.e.m. values were calculated from at least triplicates of a representative experiment. The statistical significance of differences was measured by unpaired Student’s *t*-test for experiments with two groups and a continuous outcome, or by one-way ANOVA for repeat measures. A *P* value < 0.05 indicated statistical significance. *p < 0.05, **p < 0.01, ***p < 0.00

**Results**

**TGF-beta signaling induces the expression of p16INK4a via recruitment of the Mll1 complex.**

To understand the mechanisms that mediate the recruitment of Mll1 complex to the *Ink4a/Arf* locus during replicative decline, we first used the Mouse Embryonic Fibroblasts (MEFs) model. Early passage MEFs are characterized by low p16INK4a, and high proliferation, while late passage MEFs accumulate p16INK4a, resulting in reduced proliferation (11; 18). Co-immunoprecipitation of Mll1 complex components followed by mass spectrometry suggested that Mll1 complex interacted with the TGF-beta signaling pathway effectors (data not shown). Therefore, we assessed the interaction between Mll1 complex and TGF-beta pathway using anti-Mll1 antibody immuoprecipitate from late passage (passage 9, denoted P9) MEFs, and
Western blotting using the Smad3 antibody revealed that Smad3 interacts with Mll1 (Fig. 1A and B). Using chromatin immunoprecipitations (ChIPs), we compared the binding of Smad3 to the Ink4a/Arf locus in early (passage 2, denoted P2) and late (P9) passage MEFs. Smad3 was recruited to the Ink4a/Arf locus in the late passage MEFs, suggesting a correlation between Smad3 binding and induction of p16^{INK4a} (Fig. 1C). This was further confirmed by treatment of early passage (P2) MEFs with TGF-beta, which resulted in recruitment of Smad3 to the Ink4a/Arf locus, along with Mll1 and JmjD3 (Fig. 1D; Fig. S1A), resulting in increased H3K4me3 (activating histone modification) and reduced H3K27me3 (repressive modification) levels (Fig. S1B and C). This resulted in a significant reduction in the proliferation of P2 MEFs treated with TGF-beta, due to the accumulation of p16^{INK4a} (Fig. 1E-G; Fig. S1D), which was associated with increased levels of phosphorylated Smad3 (pSmad3, Fig. S1E). Taken together, these observations suggested a novel role for TGF-beta in the transcriptional regulation of Ink4a/Arf locus.

**Inhibition of TGF-beta signaling disrupts the association of Smad3 with the Ink4a/Arf locus to promote proliferation in late passage MEFs.**

To confirm if TGF-beta signaling is associated with reduced proliferative potential through induction of p16^{INK4a} expression, we treated late passage (P9) MEFs with SB431542 (27), a well-characterized inhibitor of TGF-beta signaling that blocks phosphorylation of Smad3 via the ALK5 receptor. Treatment of P9 MEFs with SB431542 reduced the association of Smad3 (Fig. 2A), Mll1 and JmjD3 to the Ink4a/Arf locus (Fig. 2B), resulting in reduced H3K4me3 and increased H3K27me3 levels (Fig. S2, A and B). This led to reduced levels of p16^{INK4a} and increased proliferation of P9 MEFs treated with SB431542 (Fig. 2C-E, and Fig. S2C). This
suggested that indeed the decline in proliferative potential due to $p16^{INK4a}$ accumulation in late passage MEFs is mediated by TGF-beta signaling. To test if the effect of TGF-beta inhibitor on induction of proliferation was reversible, we cultured P9 MEFs in the presence of SB431542, followed by inhibitor withdrawal after 4 days of treatment. Withdrawal of SB431542 resulted in a decline in the proliferation compared to late passage MEFs that were continuously maintained in the presence of inhibitor (Fig. S2D), suggesting a transient effect of TGF-beta inhibitor on increasing proliferation.

**TGF-beta inhibition can induce beta cell replication in islets from adult mice by reducing $p16^{INK4a}$ levels.**

Based on the observations in MEFs, we examined the effect of TGF-beta signaling on regulation of the $Ink4a/Arf$ locus in islets. ChIP analysis showed increased recruitment of Smad3 to the $Ink4a/Arf$ locus in islets from 9 months old mice compared to islets from younger, juvenile mice (Fig. 3A). Treatment of pancreatic islets isolated from 9-12 months old mice with SB431542 resulted in reduced binding of Smad3, Mll1 and JmjD3 complex to the $Ink4a/Arf$ locus (Fig. 3B, and Fig. S3A), concomitant with increased H3K27me3 and reduced H3K4me3 levels at the $Ink4a/Arf$ locus (Fig. S3, B and C). We then cultured the islets from adult mice (aged 9-12 months) with TGF-beta inhibitor or vehicle control. The beta cells (marked by Pdx1) from islets treated with SB431542 showed increased replication (Fig. 3, C and D) due to reduced levels of $p16^{INK4a}$ in these islets (Fig. 3E). The specific effect of inhibition of TGF-beta signaling on beta cell replication was further confirmed by treatment of islets from aged mice with two other known TGF-beta signaling inhibitors, namely SB505124 and SD208, which showed similar effect on increased replication, while LY294002, a PI3
kinase inhibitor used as a control, did not result in any significant increase in replication (Fig. S3, D and E). Thus, treatment with small molecules inhibitors of TGF-beta signaling can promote beta cell replication in islets from adult mice that typically exhibit resistance to induction of replication and regeneration (13).

**Treatment of mice with TGF-beta inhibitor stimulates beta cell replication.**

To establish if treatment with TGF-beta inhibitor can stimulate beta cell regeneration in adult mice *in vivo*, we injected 9 months old mice intra-peritoneally with a single dose of SB431542 or vehicle control (n=9 per group). After one week the pancreas was harvested and co-immunostaining of pancreatic sections for Ki67 and insulin showed increased beta cell replication SB431542 treated mice compared to control mice (Fig. 4, A and B). The increase in beta cell proliferation was accompanied by a reduction in p16\(^{\text{INK4a}}\) levels in islets (Fig. 4C). We have previously shown that polycomb regulation of p16\(^{\text{INK4a}}\)-dependent changes that limits beta cell replication is conserved in human islets (18). We then examined whether beta cell replication can be induced in human islets by inhibition of the TGF-beta pathway. Treatment of human islets (from donors aged 40-60) with SB431542 resulted in increased beta cell replication, compared to vehicle control (Fig. 4D). The increased replication in the inhibitor treated group correlated with reduced binding of Mll1 and Smad3 at the *Ink4a/Arf* locus (Fig. S4, A and B). Thus, inhibition of TGF-beta signaling improves beta cell replication in both mice and humans.

**TGF-beta inhibitor stimulates beta cell replication in grafted human islets.**
To further evaluate the stimulatory effect of TGF-beta inhibitor on human beta-cell proliferation, we used a model wherein 1000 human islet equivalents (IEQs) were transplanted under the kidney capsule of male NOD-scid-gamma (NSG) mice (28). One week post-transplantation, mice receiving human islet grafts were treated with either the TGF-beta inhibitor (5mg/kg/BW, 50%(v/v) DMSO) or vehicle control (DMSO) while sham operated mice did not receive any treatment. Graft survival and functionality was examined by insulin secretory responses to glucose, using an ultrasensitive human insulin ELISA assay, in mice that received human islets (Fig. S5A). Detection of human insulin and C-peptide levels in the circulation 2 and 4 weeks post-transplantation indicated presence of viable human islet grafts (Fig. S5, B and C). Immunohistochemical analysis of human islet graft bearing kidney sections showed a ~10 fold (p<0.05) increase in BrdU incorporation in the TGF-beta inhibitor treated group indicating a transition from G1 to S-phase of the cell cycle (Fig. 5, A-G, Fig.S5D). The enhanced mitosis was confirmed by a ~4 fold (p<0.05) increase in Ki67 + b-cells in the treated group (Fig. 5H, Fig. S5E), and an augmentation in pH3 + beta cells suggesting progression into the G2/M phases (p=0.07) (Fig. 5I). In untreated mice, the rate of human beta-cell replication was virtually undetectable, consistent with previous findings under similar conditions (22; 23). Analysis of p16\textsuperscript{INK4a} expression in these graft samples by immunohistochemistry showed reduced p16\textsuperscript{INK4a} levels upon treatment with TGF-beta inhibitor, consistent with our earlier observations in mice (Fig. 5J). Furthermore, examination of endogenous mouse pancreas of both groups demonstrated significantly lower beta cell apoptosis (p=0.005) in TGF-β inhibitor group, compared to control mice (Fig, S6A). Despite the increased beta cell replication upon TGF-inhibitor treatment of mouse islets and human islet grafts, and decreased beta cell apoptosis in mouse islets, the beta cell area and islet density
were not different between groups (Fig. S6B, C). Thus, treatment with TGF-beta inhibitor can be effectively used to promote replication of human beta cells.

In parallel, we evaluated endogenous islets of the NSG mice that received the TGF-beta inhibitor. Consistent with our previous data in independent studies (Fig. 4B), the group that received TGF-beta inhibitors exhibited a significant increase in three different proliferation markers, namely, BrdU (0.27% ± 0.06 vs 0.06% ± 0.02, p=0.028), Ki67 (0.2% ± 0.03 vs 0.09% ± 0.01, p=0.047) and pHH3 (0.12% ± 0.02 vs 0.03% ± 0.02, p=0.039) (Fig. 6 A, B and C) in the pancreatic beta cells. Similar to human islet grafts without treatment, beta-cell replication in untreated and sham operated mice was minimal. In addition, the pancreatic beta-cell proliferation was accompanied by a reduction in p16\textsuperscript{INK4a} levels in the islets (Fig. 6D). Taken together, our studies suggest that TGF-beta signaling induces age-related accumulation of p16\textsuperscript{INK4a}, leading to replicative decline, and that treatment with TGF-beta inhibitor can effectively induce replication in both mouse and human beta cells.

**Discussion**

Previous work on TGF-beta signaling has largely focused on pancreatic development and islet homeostasis and function (29-34). Here, we delineate the mechanism by which TGF-beta signaling controls replicative senescence across cell types, including both mouse and human beta cells by regulating the Ink4a/Arf locus. More importantly, we have used small molecule inhibitors of the TGF-beta pathway to stimulate mouse and human beta cell replication \textit{in vitro} and \textit{in vivo}. TGF-beta signaling is known to inhibit cell proliferation and disruptions in TGF-
beta signaling are associated with overcoming senescence (35-37); reviewed in (38). It is important to note that we observe 2 to 3 fold increase in beta cell replication with TGF-beta inhibitors, which is comparable to the rates of replication observed during physiological beta cell mass expansion.

TGF-beta 1, 2, and 3 ligands are endogenously expressed within human pancreas, not just in islets, but also in ducts as well as the acini (3). This suggests that TGF-beta ligands may work in both autocrine and paracrine fashion in the pancreas. TGF-beta 1 levels have been reported to increase with age in circulation, as well as within several tissues (27; 28). TGF-beta signaling has been shown to be a critical signal in pancreatic development (39). Loss of TGF-beta type II receptor shows an increase in endocrine precursors and proliferating endocrine cells, suggesting that TGF beta signaling modulates the growth and differentiation of endocrine progenitors (40).

Disruptions in the canonical TGF-beta signaling in the exocrine tissue also result in islet hypertrophy (31), thus suggesting a role for TGF beta in regulating beta cell proliferation and islet homeostasis. Transient loss of Smad7 in the islets using an inducible loss of function model has been shown to result in islet enlargement (32). More recently, it has been reported that genetic loss of Smad 2 and -3 results in a more robust proliferative response upon beta cell loss (29). In another study, loss of Smad-2 was shown to induce islet hyperplasia and impaired insulin secretion (30). Studies using combined pan-pancreatic knockdown of TGF-beta receptors I and II have also implicated TGF-beta signaling in regulation of beta cell proliferation (33). However, most of such work examining the TGF-beta signaling on beta cell
proliferation has been performed in the context of adaptive expansion in permissive age windows. Our work is the first to identify a key role for TGF-beta signaling in age dependent proliferative decline in pancreatic beta cells as well as establish a mechanism of cell cycle control via INK4a locus.

TGF-beta has been reported to stimulate insulin secretion (41), insulin gene transcription and islet function (34). Further, TGF-beta has been recently reported to have a protective effect on islet grafts in the murine models (40). Also, another recent study shows that inhibition of TGF-beta signaling can reverse beta cell de-differentiation in diabetes, suggesting an effect on function (42). These studies raise the possibility that treatment with TGF-beta inhibitor may promote islet function and islet graft survival. In our studies using human islet grafts in humanized mice, however, both the control and inhibitor treated mice showed comparable graft survival and human insulin and c-peptide secretion profiles. Considering Ink4a repression by PcG proteins is a well-conserved mechanism across cell types, additional work that addresses targeting of the small molecule inhibitors of the TGF-beta signaling to the islet beta cells is warranted. Our study for the first time shows the connection between TGF-beta signaling and p16<sup>Ink4a</sup> dependent replicative senescence in beta cells, thus providing a mechanistic insight into the age dependent decline of beta cell replication. The experiments presented here establish a novel link between TGF-beta signaling and the epigenetic regulation of beta cell replicative potential. These data provide an example of a pharmacologic agent that can promote the replication of human beta cells with translational therapeutic potential to restore functional beta cell mass in patients with diabetes.
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No potential conflicts of interest relevant to this article were reported.

S.D performed the experiments and analyses on the role of TGF-beta signaling on $\text{Ink4a/Arf}$ locus in MEFs and beta cells and effect of TGF-beta inhibitor on mouse beta cell proliferation. E.D performed the experiments and analyses on the effect of TGF-beta inhibitor on human islet transplants in humanized mouse model. A.B and S.D conceived and planned the experiments and interpreted data on the role of TGF-beta signaling in MEFs and mouse beta cells, E.D and R.N.K contributed to the discussion on design, interpreted the data on human islet transplants in humanized mouse model. A.B, S.D, E.D, and R.N.K wrote the manuscript. A. B. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. We are grateful to Dr. Shuen-Ing Tschen (UCLA) for discussions, and to Emily Snyder and James Maksymetz (UCLA) for technical support.
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**Figure Legends.**

**Fig. 1.** (A) Co-immunoprecipitation analysis examining the interaction of Mll1 complex with Smad3. Cell extracts from late passage (P9) MEFs were used as input for immunoprecipitation (IP) with anti-Mll1 antibody (or control IgG) and analyzed by immunoblotting with Mll1, Jmjd3 and Smad3 antibodies. Bmi1 antibody was used as a negative control. Representative experiment from (n=3). (B) Schematic representation of the *Ink4a/Arf* locus, with blue regions, marked 1-4 indicating the amplified regions in the chromatin immunoprecipitation (ChIP) studies. (C) Chromatin immunoprecipitation (ChIP) analysis comparing the recruitment of Smad3 (using a Smad3 antibody) to the *Ink4a/Arf* locus, in early (P2) and late (P9) passage MEFs (n=3). 1-4 indicate the amplified regions at the *Ink4a/Arf* locus. 5 indicates binding for the negative control corresponding to Exon 2 in the *HoxC13* locus (43). (D) ChIP analyses comparing the recruitment of Smad3 and Mll1 to the *Ink4a/Arf* locus, in early passage (P2) MEFs treated with TGF-beta (TGFb), or vehicle control (n=3). 1-5 indicate the amplified regions, 5 being negative control. (E, F) Immunofluorescence (E), and quantification (F) for proliferation in P2 MEFs treated with TGF-beta (P2 TGFb), or vehicle control (P2C), using immunostaining with Ki67 antibody (red). DAPI (blue) is a nuclear counter-stain. Representative experiment from (n=3). (G) Western-blotting for p16 and beta-tubulin in cell extracts from P2 MEFs treated with TGF-beta (P2 TGFb), or vehicle control (P2C) (n=3). Error bars indicate ± s.e.m.; *P*-values were determined by unpaired Student’s *t*-test. *p < 0.05

**Fig. 2.** (A) ChIP analysis comparing the recruitment of Smad3 to the *Ink4a/Arf* locus, in late (P9) passage MEFs in the presence (P9 MEFs+inh) or absence of TGF-beta inhibitor (n=3). 1-5
indicate the amplified regions, 5 being negative control. 

(B) ChIP analyses comparing the recruitment of Mll1 and JmjD3 to the \textit{Ink4a/Arf} locus, in late passage (P9) MEFs treated with TGF-beta inhibitor (P9 MEFs+inh), or vehicle control (n=3). 1-5 indicate the amplified regions, 5 being negative control. 

(C, D) Immunofluorescence (C), and quantification (D) for proliferation in P9 MEFs treated with TGF-beta inhibitor (P9 Inh), or vehicle control (P9 C), using immunostaining with Ki67 antibody (red), counterstained with DAPI (blue) to mark the nuclei (n=3). 

(E) Western-blotting for p16 and beta-tubulin in cell extracts from P9 MEFs treated with TGF-beta inhibitor (P9 Inh), or vehicle control (P9 C) Representative experiment from (n=3). Error bars indicate ± s.e.m.; \(P\)-values were determined by unpaired Student’s \(t\)-test. \(^* \ p < 0.05\). 

**Fig. 3.** (A) ChIP analysis comparing the recruitment of Smad3 to the \textit{Ink4a/Arf} locus, in islets from juvenile (2.5 months) or adult (9 months) mice (n=3). 1-5 indicate the amplified regions, 5 being negative control. (B) ChIP analyses comparing the binding of Smad3 and Mll1 in islets from old (9 months) mice, treated with TGF-beta inhibitor SB431542 (inhibitor) or vehicle control (n=3). 1-5 indicate the amplified regions, 5 being negative control. 

(C, D) Immunohistochemistry (C), and quantification (D) for proliferation of beta cells in islets from 9 months old mice, treated with TGF-beta inhibitor, or vehicle control, using immunostaining with Ki67 antibody (red), and beta cell marker Pdx1 (green). Counterstain DAPI (blue) marks the nuclei (n=3). 

(E) Western-blotting for p16 and beta-tubulin in extracts from islets from 9 months old mice, treated with TGF-beta inhibitor, or vehicle control. Representative experiment from (n=3). Error bars indicate ± s.e.m.; \(P\)-values were determined by unpaired Student’s \(t\)-test. \(^* \ p < 0.05; ** \ p < 0.01\).
Fig. 4. (A, B) Immunohistochemistry (A), and quantification (B) for proliferation of beta cells in pancreatic sections from 9 months old mice, injected intra-peritoneally with TGF-beta inhibitor, or vehicle control, using immunostaining with Ki67 antibody (red), and insulin (green), along with nuclear counterstain DAPI (blue). Arrows mark the proliferating beta cells, labeled by Ki67 and insulin (n=9). (C) Immunohistochemistry for p16\textsuperscript{INK4a} levels in pancreatic sections from 9 months old mice, injected intra-peritoneally with TGF-beta inhibitor, or vehicle control, using immunostaining with p16\textsuperscript{INK4a} antibody (red), and insulin (green), along with DAPI (blue) Representative experiment from (n=9). (D) Immunohistochemistry for Ki67 (red), along with insulin (green), and DAPI (blue) for proliferation of beta cells in human islets treated with TGF-beta inhibitor, or vehicle control. Arrowheads mark proliferating beta cells. Representative experiment from (n=3). Error bars indicate ± s.e.m.; P-values were determined by unpaired Student’s t-test. *p < 0.05

Fig. 5. (A-F) Confocal microscopy view of kidney sections derived from grafted human islets under the kidney capsule and treated with TGF-beta inhibitor. Magnified area highlighted from A (right white box) shows imaging of insulin (red) (B,D and F), BrdU antibody (green) (C,D and F), and DAPI (blue) (E and F). Arrows mark proliferating beta cells. Magnified images (60x) show each immunofluorescent staining separately (indicating nuclear gap for replicating beta cell surrounded by insulin) for the proliferating beta-cells. 25x and 60X magnification. Representative experiment from (n=4). (G-I) Quantification of proliferating beta cells for (G) BrdU/insulin, (H) Ki67/insulin and (I) pHH3/insulin double positive cells from human islet grafted and TGF-beta inhibitor treated groups. Between 1000 to 2000 beta cells were counted in each section (n=4). (J) Immunohistochemistry for p16\textsuperscript{INK4a} in human islet grafted kidney
sections treated with or without TGF-beta inhibitor, using insulin (red), p16 (green) and DAPI (blue) Representative experiment from (n=4). Error bars indicate ± s.e.m.; P-values were determined by unpaired Student’s t-test. *p < 0.05 and **p < 0.01

Fig. 6. (A-D) Immunohistochemistry and quantification for proliferating beta-cells in pancreatic sections of human islet graft bearing mice. H&E staining (A) and immunoflourescent staining for proliferation markers (B) BrdU, (C) Ki67 and (D) pH3. All proliferation markers are shown in green, insulin in red and nuclear staining in blue. Arrows mark proliferating beta cells. Representative experiment from (n=4). (E) Immunohistochemistry for p16

ink4a in pancreatic section from human islets grafted recipients treated with or without TGF-beta inhibitor, using insulin (red), p16 (green) and DAPI (blue) Representative experiment from (n=4). Error bars indicate ± s.e.m.; P-values were determined by unpaired Student’s t-test. *p < 0.05.
Fig. 2.

A

B

C

D

E

105x66mm (300 x 300 DPI)
Supplemental Figures.

Fig. S1. (A, B, C) ChIP analyses comparing the recruitment of JmjD3 (A) and levels of H3K27me3 (B) and H3K4me3 (C) at the Ink4a/Arf locus, in early passage (P2) MEFs treated with TGF-beta (TGFb), or vehicle control. 1-5 indicate the amplified regions, 5 being negative control. (D) Cell doubling in P2 MEFs upon treatment with TGF-beta (TGFb), or vehicle control. (E) Western-blotting for p-Smad3 and beta-tubulin in extracts from islets from 9 months old mice, treated with TGF-beta inhibitor, or vehicle control. Representative experiment from (n=3). Error bars indicate ± s.e.m.; P-values were determined by unpaired Student’s t-test. The analyses show a representative from at least three independent experiments. *p < 0.05; ** p<0.01
**Fig. S2.** (A, B) ChIP analyses comparing the levels of H3K27me3 (A) and H3K4me3 (B) at the Ink4a/Arf locus, in late passage (P9) MEFs treated with TGF-beta inhibitor (inh), or vehicle control. 1-5 indicate the amplified regions, 5 being negative control. (C) Cell doubling in P9 MEFs upon treatment with TGF-beta inhibitor (Inh), or vehicle control (N=1). (D) Cell doubling in P9 MEFs upon continuous treatment with TGF-beta (Inh), or after inhibitor withdrawl after day 4 (Inh w), or vehicle control (C) (N=1). Error bars indicate ± s.e.m.; *P*-values were determined by unpaired Student’s *t*-test. *p* < 0.05; **p** < 0.01 The analyses show a representative from at least three independent experiments, unless otherwise indicated.
**Fig. S3.** (A, B, C) ChIP analyses comparing the recruitment of JmjD3 (A) and levels of H3K27me3 (B) and H3K4me3 (C) at the **Ink4a/Arf** locus, in islets from old (9 months) mice, treated with TGF-beta inhibitor, or vehicle control. 1-5 indicate the amplified regions, 5 being negative control. (D, E) Immunohistochemistry (D), and quantification (E) for proliferation of beta cells in islets from 9 months old mice, treated with indicated TGF-beta inhibitors (SB431542, SB505124, SD208, or a control small molecule LY294002, using immunostaining with Ki67 antibody (red), and beta cell marker Pdx1 (green). (F, G) Immunohistochemistry (F), and quantification (G) for proliferation of beta cells in islets from 9 months old mice, treated with **Alk5** siRNA, or control, scrambled.
siRNA, using immunostaining with ki67 antibody (red), and beta cell marker Pdx1 (green). Error bars indicate ± s.e.m.; P-values were determined by unpaired Student’s t-test. *p < 0.05; ** p<0.01. The analyses show a representative from at least three independent experiments (N=3), unless otherwise indicated.
Fig. S4. (A, B) ChIP analyses comparing the recruitment of Mll1 (A) and Smad3 (B) at the Ink4a/Arf locus, in human islets from adult cadaveric donors, treated with TGF-beta inhibitor, or vehicle control. 1-5 indicate the amplified regions, 5 being negative control. Error bars indicate ± s.e.m.; P-values were determined by unpaired Student’s t-test. *p < 0.05. The analyses show a representative from at least three independent experiments (N=3), unless otherwise indicated.
Fig. S5. (A) Acute (first)-phase human insulin secretion (following an intraperitoneal injection of glucose, 3g/Kg body weight) from human islets grafted under the kidney capsule of NSG mice, treated with or without TGF-beta inhibitor. Data are expressed as increase in human insulin at 2 (T2) and 5 (T5) minutes, relative to time 0 (T0). (B, C) Serum Human insulin (B) and human C-peptide (C) levels. To visualize control group
(sham operated mice) in the human insulin and C-peptide graphs we used representative values. Representative experiments from (n=4). Error bars indicate ± s.e.m. *P-values were determined by unpaired Student’s t-test. *p < 0.05. (D) Immunohistochemistry of human islet transplanted (top panels) and human islet transplanted and treated with TGF-beta inhibitor (bottom panels), kidney sections stained with BrdU antibody (green), insulin (red) and DAPI (blue). Arrows mark replicating beta cells. Magnified images show each immunofluorescent staining separately (indicating nuclear gap for replicating beta cell surrounded by insulin) for the proliferating beta-cells. 10x and 40X magnification. Representative experiment from (n=4). (E) Immunohistochemistry of human islet transplanted and treated with TGF-beta inhibitor (bottom panels), kidney sections stained with Ki67 antibody (green), insulin (red) and DAPI (blue). Arrows mark replicating beta cells. Magnified images show each immunofluorescent staining separately (indicating nuclear gap for replicating beta cell surrounded by insulin) for the proliferating beta-cells.
**Fig. S6**

(A) TUNEL staining (left panels), and quantification (right panel, graph) of paraffin-embedded sections of pancreatic tissue obtained from mice receiving twice/week TGF beta inhibitor treatment for 3 weeks 7 days after human islet transplantation. Scale bar 200 µm. Arrows indicate TUNEL⁺ beta cells undergoing apoptosis. Inset shows a magnified representative image of TUNEL⁺ beta cell. (B) Morphometric analysis (left panels), and quantification (right panel, graph) of beta cell area of pancreatic sections from DMSO and TGF-beta inhibitor treated mice. (C) Representative pancreas sections (magnification x20; left panels) showing insulin (red) positive islets. Nuclear staining by DAPI is shown in blue. The right panel (graph) shows total beta cells per mm² pancreatic area. *n*=4 mice each group and **P<0.05 (student t-test). Data are expressed as mean±SEM.