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PDGF signalling controls age-dependent proliferation in pancreatic β -cells

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Abstract

Determining the signalling pathways that direct tissue expansion is a principal goal of regenerative biology. Vigorous pancreatic β -cell replication in juvenile mice and humans declines with age, and elucidating the basis for this decay may reveal strategies for inducing β -cell expansion, a long-sought goal for diabetes therapy. Here we show that platelet-derived growth factor receptor (Pdgfr) signalling controls age-dependent β -cell proliferation in mouse and human pancreatic islets. With age, declining β -cell Pdgfr levels were accompanied by reductions in β -cell enhancer of zeste homologue 2 (Ezh2) levels and β -cell replication. Conditional inactivation of the *Pdgfra* gene in β -cells accelerated these changes, preventing mouse neonatal β -cell expansion and adult β -cell regeneration. Targeted human PDGFR- α activation in mouse β -cells stimulated Erk1/2 phosphorylation, leading to Ezh2-dependent expansion of adult β -cells. Adult human islets lack PDGF signalling competence, but exposure of juvenile human islets to PDGF-AA stimulated β -cell proliferation. The discovery of a conserved pathway controlling age-dependent β -cell proliferation indicates new strategies for β -cell expansion.

Acquired tissue insufficiency underlies the pathogenesis of diverse human diseases; for example, absolute or relative deficits in pancreatic β -cells underlie diabetes mellitus. To expand insulin-secreting β -cells, investigators have modulated intracellular factors, including β -cell cycle regulators, or activated intrinsic regulatory pathways with extrinsic

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mitogenic factors^{1,2}. In mice or other experimental animals, β -cell expansion from such efforts has been transient or limited, and often complicated by uncontrolled β -cell growth, loss of defining β -cell features and impaired metabolic control^{3,4}. Likewise, induction of human islet β -cell proliferation remains an unattained goal, with studies reporting inconsistent mitogenic responses^{5,6}. Thus, we sought to identify native signalling pathways governing physiological β -cell proliferation and regeneration.

During physiological growth in humans, mice and other species, juvenile β -cells expand by self-renewal^{7,8} while maintaining their hallmark functions. Pancreatic β -cell proliferation decreases rapidly in juvenile mice and humans, then declines more slowly in adults^{9,10}. Studies indicate that age-dependent increases of p16^{INK4a}, a cyclin-dependent kinase inhibitor encoded by *Cdkn2a*, restrict proliferation of mouse and human β -cells and other tissues with ageing^{11,12}. We showed that the polycomb group protein Ezh2 represses *Cdkn2a* and promotes β -cell proliferation in juvenile mouse islets¹³. Ezh2 is a histone methyltransferase crucial for trimethylation of histone H3 on Lys 27 (H3K27me3), a modification mediating transcriptional repression^{14,15}. *Ezh2* expression in mouse and human β -cells declines rapidly in juveniles, then slowly in adults¹³, similar to the tempo of age-dependent decline in β -cell proliferation^{9,10}. Conditional *Ezh2* inactivation in β -cells accelerated loss of H3K27me3 repression at *Cdkn2a*, resulting in premature p16^{INK4a} expression, impaired β -cell proliferation, reduced β -cell mass and inadequate β -cell regenerative recovery after β -cell ablation¹³. We proposed that conserved regulatory pathways governing *Ezh2* expression in ageing islets could be targeted to stimulate adult β -cell proliferation.

Pdgfr signalling promotes proliferation, survival and migration in diverse cell types¹⁶. Pdgfr signalling activation stimulates DNA synthesis in cultured islets^{17,18}, but the roles of Pdgfr signalling in β -cell proliferation and expansion were unknown. Pdgf-induced actin polymerization in fibroblasts requires Ezh2 (ref. 19), suggesting that Pdgf signalling might regulate *Ezh2* expression and age-dependent β -cell proliferation in islets. Here we demonstrate that a pathway regulated by Pdgfr- α , governs the expression and activity of intrinsic β -cell factors—including the retinoblastoma protein (Rb), E2f transcription factors and Ezh2—to control β -cell proliferation. Modulation of β -cell Pdgf signalling attenuates age-dependent proliferative failure and Ezh2 loss, permitting *in vivo* expansion of β -cells with regulated function. Cardinal features of this pathway are conserved in human islets, permitting conditional induction of human β -cell replication.

Age-dependent β -cell Pdgfr- α loss

To investigate whether Pdgf signalling might regulate islet β -cell proliferation and *Ezh2* expression, we assessed Pdgf receptor and ligand expression in islets and β -cells. Immunohistology revealed that Pdgfr- α and Pdgfr- β levels were markedly reduced in islet β -cells at 6 weeks and 6 months of age, compared to neonatal islets (Fig. 1a and Supplementary Fig. 1a). Real-time polymerase chain reaction with reverse transcription (RT-PCR) studies of β -cells purified by fluorescence-activated cell sorting (FACS) confirmed that *Pdgfra*, *Pdgfrb* and *Ezh2* messenger RNA levels were reduced in adult β -cells at 5 months compared to 2-week-old β -cells (Fig. 1b). Further RT-PCR analyses of isolated islets from wild-type mice ranging from 2 weeks to 13 months revealed an age-dependent decline of islet mRNAs encoding *Pdgfra*, *Pdgfrb* and Pdgf ligands (Fig. 1c and Supplementary Fig. 1b–e). In contrast, islet mRNAs encoding the insulin receptor, prolactin receptor, glucocorticoid receptor and intrinsic regulators of β -cell proliferation like *Pdx1*, *Foxo1*, *Foxo3*, *Rb1* (hereafter referred to as *Rb*), *p130* (also known as *Rbl2*) and *E2f1*, did not decline in mice with advancing age (Supplementary Fig. 2). Thus, reduced β -cell levels

of Pdgfr receptors and ligands corresponded with *Ezh2* loss and age-dependent reduction of β -cell replication¹³.

To assess whether premature Pdgfr loss might compromise *Ezh2*-dependent neonatal β -cell proliferation, we generated *RIP-Cre; Pdgfra^{fl/f}* mice (abbreviated to β PdgfraKO) that permit conditional *Pdgfra* inactivation in islet β -cells and loss of detectable *Pdgfra* mRNA and protein expression (Fig. 1d and Supplementary Fig. 3a). Compared to controls, β -cell *Ezh2* protein and islet *Ezh2* mRNA levels were reduced whereas *p16^{INK4a}* mRNA levels were increased in β PdgfraKO islets (Fig. 1e and Supplementary Fig. 3a, b); in contrast, *Pdgfrb* and *Ezh1* mRNA levels were unchanged in β PdgfraKO islets (Supplementary Fig. 3c, d). In 2–3-week-old β PdgfraKO mice, we detected a threefold reduction in the β -cell proliferation rate and a 50% reduction in β -cell mass (Fig. 1f, g). β PdgfraKO mice at 3 weeks had mild hyperglycaemia during *ad libitum* feeding and impaired glucose tolerance in a glucose challenge test (Supplementary Fig. 3e, f). Thus, premature loss of *Pdgfra* impairs β -cell proliferation and expansion and glucose control in young mice.

Pdgfr- α controls adult β -cell regeneration

Destruction of β -cells by streptozotocin (STZ) in mice provokes β -cell proliferation with regeneration of β -cell mass^{11,13}. Following STZ exposure, *Pdgfra* and *Pdgfrb* were induced in wild-type islets (Fig. 2a, b), suggesting possible roles for Pdgfr signalling in adult β -cell regeneration. Consistent with this view, β -cell mass in control mice gradually recovered after STZ treatment, accompanied by increased β -cell *Ezh2* expression and proliferation; in contrast, β PdgfraKO β -cells failed to increase *Ezh2* or proliferation to restore β -cell mass (Fig. 2c–e). β PdgfraKO mice developed severe diabetes that persisted throughout the course of our studies (Fig. 2f). In contrast, control mice became moderately diabetic immediately after STZ challenge, but achieved restored glucose control 3–4 weeks thereafter (Fig. 2f), coinciding with increases of β -cell mass (Fig. 2e). Thus, Pdgfr- α is also required in adult β -cells for compensatory proliferation in experimental diabetes.

PDGFR- α promotes functional β -cell expansion

To test whether enhanced Pdgfr signalling might promote β -cell expansion, we exposed islets from juvenile and adult mice to physiological concentrations of platelet-derived growth factor-AA (PDGF-AA). Exposure of 3-week-old islets to PDGF-AA increased phospho-Pdgfr- α levels and *Ezh2* mRNA and protein levels, but not *Ezh1* expression (Supplementary Fig. 4a–c). In contrast, juvenile islets exposed to mitogens like insulin or prolactin did not detectably alter *Ezh2* mRNA levels (Supplementary Fig. 4d, e). Unlike in 3-week-old islets, levels of *Ezh2* mRNA and protein were not significantly increased in 7- or 9-month-old adult islets exposed to PDGF-AA (Supplementary Fig. 4c, f). PDGF-AA exposure increased β -cell proliferation in juvenile islets, as assessed by BrdU incorporation, but not in adult islets (Supplementary Fig. 4g, h). Increased *Ezh2* expression and β -cell BrdU incorporation were eliminated by simultaneous treatment with the receptor tyrosine kinase inhibitors Sunitinib²⁰ or Vargatef²¹ (Supplementary Fig. 4a, c, g, h). Thus, PDGF-AA exposure was sufficient to stimulate β -cell replication in juvenile islets, but loss of competence for Pdgfr prevented this response to PDGF-AA in adult islets.

To investigate whether sustained PDGFR signalling permitted adult β -cell expansion *in vivo*, we intercrossed the *RIP-Cre* strain with mice harbouring a human *PDGFRA*^{D842V} allele encoding a ligand-independent activated receptor inserted at the *ROSA26* (*R26*) locus²². A *loxP*-flanked transcriptional stop sequence permits Cre-dependent *PDGFRA*^{D842V} expression and activation of PDGF signalling *in vivo*²². In contrast to littermate controls, mice with the *RIP-Cre; R26-PDGFRA*^{D842V} genotype (abbreviated to β PDGFR α Tg) expressed human *PDGFRA* mRNA and PDGFR- α (D842V) protein in islet β -

cells (Fig. 3a and Supplementary Fig. 5a). Expression of *R26-PDGFRα^{D842V}* did not affect endogenous islet *Pdgfra* mRNA levels (Fig. 3a) or β-cell expansion and glucose control in neonatal or juvenile βPDGFRαTg mice (Fig. 3b, c and Supplementary Figs 5b and 6a–d). By 3 months, β-cell *Ezh2* expression had declined in control mice, but β-cell *Ezh2* expression in βPDGFRαTg mice was maintained at elevated levels, a difference that persisted for 8–14 months (Fig. 3e, f). mRNA levels of islet *p16^{INK4a}* and *p19^{Arf}*, both targets¹³ of *Ezh2*, were reduced in βPDGFRαTg mice compared to those in littermate controls (Supplementary Fig. 6f, g). In contrast, islet mRNA levels of *Ezh1* in βPDGFRαTg and control mice were indistinguishable (Supplementary Fig. 6h). β-cell proliferation in 14-month-old βPDGFRαTg mice was increased ninefold compared to age-matched controls, a level of proliferation seen in 3-month-old control β-cells (Fig. 3d, f). β-cell mass was increased in βPDGFRαTg mice at 3 months, and remained increased at 14 months compared to age-matched controls (Fig. 3b). In contrast, total pancreatic mass and islet architecture in βPDGFRαTg mice and controls were indistinguishable (Supplementary Figs 5c and 6b). Thus, *Pdgfr* signalling activation is sufficient to sustain adult β-cell expansion *in vivo*.

Prior studies indicate that β-cell expansion stimulated by mitogens can be complicated by disrupted β-cell function and impaired metabolic control^{3,4}. To assess the physiological impact of prolonged adult β-cell proliferation in βPDGFRαTg mice, we measured systemic blood glucose and insulin levels. Blood glucose levels during fasting and *ad libitum* feeding and responses to insulin challenge were indistinguishable in βPDGFRαTg mice and littermate controls (Supplementary Fig. 6c–e). During re-feeding after overnight fast, βPDGFRαTg mice had a 25% reduction of blood glucose (Fig. 3g), associated with a modest elevation of plasma insulin levels (Supplementary Fig. 6i), and both changes were sustained for up to 14 months. After overnight fast, glucose clearance was enhanced in glucose-challenged 3- or 14-month-old βPDGFRαTg mice (Fig. 3h and Supplementary Fig. 6j). Thus, PDGFRαTg mice maintained normal glucose-regulated insulin release and glycaemic control, suggesting that β-cell function remained regulated in these mice.

PDGF-induced β-cell expansion requires *Ezh2*

To test whether PDGFR-α-induced β-cell proliferation and expansion required *Ezh2*, we generated mice permitting conditional inactivation¹³ of *Ezh2* in βPDGFRαTg β-cells. Intercrosses generated *RIP-Cre; Ezh2^{fl/f}; R26-PDGFRα^{D842V}* mice (abbreviated to βEzh2KO-RαTg) and littermate or sibling *RIP-Cre; Ezh2^{fl/+}; R26-PDGFRα^{D842V}* mice (abbreviated to βEzh2HET-RαTg) and *RIP-Cre; Ezh2^{fl/f}* mice (abbreviated to βEzh2KO). Islet *PDGFRα^{D842V}* mRNA levels in both βEzh2KO-RαTg and βEzh2HET-RαTg mice were elevated and indistinguishable (Fig. 4a). By contrast, increased islet *Ezh2* mRNA levels and β-cell *Ezh2* protein were not observed in βEzh2KO-RαTg or βEzh2KO littermates (Fig. 4b and Supplementary Fig. 7), confirming Cre-mediated deletion of *Ezh2* in these mice. As expected, β-cell BrdU incorporation and mass were increased in βEzh2HET-RαTg mice (Fig. 4c, d and Supplementary Fig. 7) compared to controls (either *Ezh2^{fl/f}; R26-PDGFRα^{D842V}* or *Ezh2^{fl/+}; R26-PDGFRα^{D842V}* genotype). However, neither β-cell BrdU incorporation nor mass were increased in βEzh2KO-RαTg mice compared to controls (Fig. 4c, d and Supplementary Fig. 7). Consistent with these findings, we detected modest postprandial blood glucose reduction and increased blood insulin levels in βEzh2HET-RαTg mice, but not in βEzh2KO-RαTg mice (Fig. 4e, f). Thus, *Ezh2* is required for β-cell expansion and metabolic changes from PDGFR-α activation in β-cells.

β -cell PDGFR controls *Ezh2* via Erk and Rb/E2f

To elucidate the signalling basis of altered β -cell proliferation in β PDGFR α Tg, β PdgfraKO and ageing wild-type mice, we investigated Pdgf signal transduction factors. Pdgf receptors activate signalling elements¹⁶, including the mitogen-activated protein kinase/extracellular signal-regulated kinase (Mapk/Erk), phosphatidylinositol 3-kinase (PI3K)/Akt and phospholipase PLC- γ . *Ezh2* induction in juvenile wild-type islets exposed to PDGF-AA was blocked by U0126, which inhibits Erk1/2 activation²³, but not by LY294002 or U-73122, inhibitors of PI3K or PLC- γ signalling (Supplementary Fig. 8a). Similarly, increased β -cell BrdU incorporation in islets exposed to PDGF-AA was blocked by U0126, but not by LY294002 (Supplementary Fig. 8b), indicating that Erk mediates β -cell Pdgf signalling responses. We also detected increased phosphorylation of Erk1/2, but not phosphorylation of Akt or PLC- γ , in β PDGFR α Tg islets compared to control islets (Fig. 5a, b). Immunohistology confirmed increased phospho-Erk1/2 levels in β -cells of β PDGFR α Tg islets compared to controls (Fig. 5c). PDGF/ERK signalling activates cell cycle regulators to stimulate proliferation^{24,25}, and β PDGFR α Tg islets had elevated levels of *Ccnd1* mRNA encoding cyclin D1, and increased β -cells with nuclear cyclin D1 and phosphorylated Rb protein (Fig. 5c–e and Supplementary Fig. 9a). In contrast, loss of β -cell Pdgf signalling in β PdgfraKO mice diminished islet levels of *Ccnd1* mRNA, and reduced numbers of phospho-Erk1/2⁺ and phospho-Rb⁺ β -cells (Supplementary Fig. 9b, c). Rb protein phosphorylation regulates association of the E2f1 transcriptional activator and E2f4 transcription repressor with their targets^{26,27}. We identified two conserved candidate E2f-binding elements near the *Ezh2* promoter (Supplementary Fig. 10), and chromatin immunoprecipitation (ChIP) studies showed increased E2f1 association and reduced E2f4 association with these *Ezh2* elements in β PDGFR α Tg islets (Fig. 5f, g). In wild-type mice, levels of phospho-Erk1/2 and phospho-Rb declined with age in islet β -cells at a tempo similar to declining *Ezh2* and *Pdgfra* expression (Supplementary Fig. 11a, b). ChIP studies also revealed that association of E2f1 with *Ezh2* decreased, whereas association of E2f4 with *Ezh2* elements increased in islets with age (Supplementary Fig. 11c, d). Thus, age-dependent attenuation of β -cell Pdgfr- α altered Erk and Rb/E2f signalling, resulting in reduced *Ezh2* expression.

To verify that Rb/E2f signalling regulates β -cell *Ezh2*, we examined triple mutant *ROSA-CreERT2*; *Rb*^{f/f}; *p130*^{f/f}; *p107*^{-/-} mice (RbTriKO) lacking Rb family proteins²⁸. After exposure to tamoxifen, β -cells in adult RbTriKO mice, lacking detectable Rb, p130 and p107, had increased *Ezh2* protein and mRNA levels, compared to those from tamoxifen-exposed *Rb*^{f/f}, *p130*^{f/f}, *p107*^{-/-} control littermates (Supplementary Fig. 12a–c). Islet ChIP studies revealed increased E2f1 association, and reduced E2f4 association with *Ezh2* cis-regulatory elements (Supplementary Fig. 12d, e). Consistent with these findings, BrdU incorporation in RbTriKO β -cells was increased by 15-fold, culminating in a 400% increase of β -cell mass and hypoglycaemia that progressively worsened until death (Supplementary Fig. 12f–h). Thus, Rb proteins constrain *Ezh2* expression and adult islet cell growth.

PDGF control of human β -cell proliferation

To investigate the relevance of our studies to human β -cells, we assessed PDGF signalling and competence in human islets. Like in juvenile mice, PDGFR- α protein and phospho-ERK1/2 levels were readily detected in islets from young human donors (Fig. 6a, b). We also detected abundant phospho-RB-Ser 780 and EZH2 protein in β -cell nuclei from young islets (Fig. 6b). In contrast, PDGFR- α was only detected in non- β -cells of islets from adult human donors, with little to no detectable phospho-ERK1/2, phospho-RB or EZH2 in β -cells (Fig. 6a, b), suggesting that PDGF signalling attenuation is a conserved feature of ageing islet β -cells. mRNA levels of *EZH2*, but not *EZH1*, were increased in juvenile human islets exposed to PDGF-AA, an effect blocked by Sunitinib or U0126 (Fig. 6c and

Supplementary Fig. 13a). In contrast, basal *EZH2* mRNA levels were lower in adult islets and not induced by PDGF-AA (Fig. 6c). Similar to juvenile mouse islets with activated Pdgfr signalling, ChIP studies demonstrated PDGF-AA-stimulated association of E2F1 with the human *EZH2* locus, an effect blocked by Sunitinib (Fig. 6d). Thus, evolutionarily conserved elements of the PDGF signalling pathway govern *EZH2* expression and β -cell cycle regulators in human islets. Proliferating BrdU⁺ cells in juvenile islets stimulated by PDGF-AA were predominantly β -cells, verified by immuno-localization of BrdU⁺ nuclei within insulin⁺ cells and PDX1⁺ nuclei with confocal microscopy (Fig. 6f and Supplementary Fig. 13b, c). Compared to vehicle-exposed controls, juvenile islets exposed to PDGF-AA had a sixfold increase of β -cell BrdU incorporation, an effect eliminated by simultaneous exposure to Sunitinib or U0126 (Fig. 6e, f). In contrast, PDGF-AA did not alter β -cell proliferation in adult human islets (Fig. 6e, f). Thus, dynamic PDGF signalling competence may regulate declining β -cell proliferation in ageing human islets.

Discussion

The elucidation and control of mechanisms governing pancreatic β -cell proliferation could transform treatments for diseases like diabetes. However, prior attempts to expand islets by modulating extrinsic or intrinsic growth regulators have been bedevilled by limited proliferation with loss of defining β -cell features^{3,4}, indicating disruption of mechanisms that preserve fate in proliferating β -cells. The maintenance of cell fate and function in proliferating cells is the essence of epigenetic regulation. Here we focused on identifying the principal elements of a native signalling pathway regulating *Ezh2*, an essential epigenetic regulator of β -cell proliferation¹³. We found that β -cell Pdgfr signalling was required for *Ezh2* induction, to sustain both physiological and regenerative β -cell proliferation.

During islet β -cell regeneration (this study), and in maternal islets in pregnancy (H.C. and S.K.K., unpublished results), we observed a twofold increase of islet *Ezh2* mRNA levels. However, pathological loss of all Rb family proteins in RbTriKO islets led to a fourfold increase in *Ezh2* mRNA, and a 15-fold increase in β -cell proliferation. RbTriKO mice became hypoglycaemic and developed other islet and metabolic phenotypes reminiscent of insulinoma pathogenesis. Thus, the physiological expansion of β -cells maintaining regulated function may require mechanisms limiting *Ezh2* induction. Compared with β -cells from β Ezh2KO mice, we also observed a modest enhancement of proliferation and expansion after PDGFR- α activation in β -cells lacking *Ezh2*. Insulin or prolactin, two mitogens capable of promoting β -cell proliferation^{1,2,29}, did not induce β -cell *Ezh2* expression in cultured islets. Thus, our findings here support the view that *Ezh2*-independent mechanisms regulate β -cell proliferation. Likewise, decline of β -cell proliferation in ageing β PDGFR α Tg mice suggests that PDGF-independent pathways restrict β -cell expansion.

Proliferative responses to PDGF decline in some ageing cells³⁰, and progenitor cell self-renewal is impaired by Pdgfr signalling disruption³¹. However, the molecular basis for these observations and the roles of Pdgfr signalling in islet β -cell growth and development were not established^{17,18,32}. Here we showed that loss of β -cell Pdgfr- α signalling accelerated β -cell replication failure both in juvenile mice and in adult mice after conditional β -cell ablation. *In vivo* activation of β -cell PDGFR- α signalling increased *Ezh2* expression and mitigated age-dependent decline of β -cell proliferation. Crucially, conditional *Ezh2* inactivation in β -cells with activated PDGFR- α signalling prevented these changes. Thus, our conditional loss- and gain-of-function studies reveal how Pdgfr signalling regulates age-dependent physiological β -cell proliferation and expansion. Our work also suggests that Mapk/Erk regulation links Pdgfr activation to *Ezh2* induction and β -cell proliferation, consistent with studies demonstrating Erk regulation of *Ezh2* in cancer cells³³, and Erk activation that accompanies mouse β -cell expansion in pregnancy³⁴. Further studies should

clarify how Erk and other factors might integrate β -cell responses to Pdgf or other mitogens³⁵.

Although the mechanisms underlying pancreatic β -cell expansion in physiological settings may differ between species^{36,37}, age-dependent restriction of β -cell proliferation is a conserved feature in mice and humans^{9,10}. Here, we provide unprecedented evidence that a conserved signalling pathway restricts β -cell proliferation in mice and humans. We suggest that β -cell competence for signalling factors like PDGF can underlie dynamic islet responses to mitogens. Such age-dependent signalling competence might explain earlier findings of inconsistent human β -cell responses to other mitogens^{5,6}. PDGF ligands are measurable in serum, and regulation of such circulating signalling agonists may provide an additional level of growth control in competent islet β -cells. Prior studies correlated increased *PDGFR* and *EZH2* expression in human endocrine neoplasias³⁸, and disrupted PDGF signalling in β -cells of humans with type 2 diabetes³⁹. Thus, our demonstration that PDGF signalling regulates *EZH2*, *CDKN2A* and β -cell proliferation in human islets combines several observations into a new molecular model for physiological and pathological β -cell growth. The factors governing β -cell expression of PDGF signalling regulators like PDGFR- α remain unknown, but we speculate that the identification of such factors may be useful for modulating human β -cell growth and function in diabetes and cancer.

METHODS SUMMARY

Animal studies and mouse islet culture experiments

Details for various mouse strain generation, *in vivo* manipulation and measurement, mouse islet isolation and culture and FACS-based β -cell purification are provided in Supplementary Data.

Histology, immunohistochemistry and molecular studies

Standard histology and immunostaining protocols for various proteins on pancreas or islet sections were described previously¹³. β -cell mass and proliferation were morphometrically quantified¹³ by independent researchers blinded to sample identity. Details of these studies and real-time RT-PCR, western blot and ChIP analyses are provided in Supplementary Data.

Human pancreas and islet studies

Four juvenile and four adult pancreases, and eight juvenile and three adult individual islet preparations from healthy human deceased organ donors (mean age 3.0 ± 0.6 years for juveniles; mean age 44.3 ± 5.3 years for adults) were used in this study. As in the mouse studies, similar procedures and experiments were performed on human pancreatic sections or islet cultures as described in Supplementary Data.

Statistical analyses

Results are shown as mean $\pm$ standard error of the mean (s.e.m.). Statistical analysis was performed by one-way ANOVA, two-way repeated measures ANOVA, or unpaired Student's *t*-test, as appropriate, with significance set at $P < 0.05$.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

METHODS

Animals

Transgenic mice harbouring a floxed *Pdgfra* allele⁴⁰ encoding Pdgfr- α were purchased from the Jackson Laboratory. This strain of mice was intercrossed with *RIP-Cre* mice⁴¹ expressing Cre recombinase from rat insulin 2 gene promoter elements to generate *RIP-Cre*, *Pdgfra*^{f/f} mice (designated as β PdgfraKO) and their littermate controls (including *RIP-Cre*, *Pdgfra*^{f/+} mice designated as β PdgfraHet) on a mixed 129/Sv; C57BL/6 genetic background.

R26^{hPDGFR α PM} transgenic mice that harbour a mutated human *PDGFRA*^{D842V} allele encoding a ligand-independent activated receptor targeted immediately after a *loxP*-flanked transcriptional stop sequence at the *ROSA26* locus have been described previously²². This strain of mice was intercrossed with *RIP-Cre* mice to generate *RIP-Cre*; R26-*PDGFRA*^{D842V} mice (designated as β PDGFR α Tg) together with their littermate controls on a mixed 129/Sv; C57BL/6 genetic background. Mice with specific expression of mutant human *PDGFRA*^{D842V}, but lacking *Ezh2* in β -cells (designated as β Ezh2KO-R α Tg), were generated from subsequent intercrosses between β Ezh2KO¹³ and β PDGFR α Tg mice.

Tamoxifen-inducible, compound triple-mutant mice lacking retinoblastoma pocket proteins Rb, p130 and p107 (*ROSA26-CreERT2*, *Rb*^{f/f}, *p130*^{f/f}, *p107*^{-/-}, designated as RbTriKO) were produced in the Sage group (Stanford University). RbTriKO mice harbour a germline-deleted *p107* null allele (*p107*^{-/-}), and *loxP*-flanked *Rb* and *p130* conditional alleles which can be excised by tamoxifen-sensitive CreER^{T2} recombinase targeted into the *ROSA26* locus²⁸. Littermate mice (*Rb*^{f/f}, *p130*^{f/f}, *p107*^{-/-}) lacking *ROSA26-CreERT2* were used as controls. To delete *Rb* and *p130* alleles, RbTriKO and littermate control mice aged 3–5 months received intraperitoneal injections of tamoxifen (Sigma, 1.5 mg per mouse per day) dissolved in ethanol/corn oil (Sigma) on five consecutive days.

MIP-EGFP mice⁴² were obtained from the laboratory of M. Hara. C57BL/6 mice were purchased from the National Cancer Institute (NCI). All mutant mice used in this study were genotyped by PCR of tail genomic DNA for the human *PDGFRA*^{D842V} allele, mouse *Pdgfra*^f, *Ezh2*^f, *Rb*^f, *p130*^f, *p107*⁻ alleles, and transgenes encoding EGFP, *RIP-Cre* or *ROSA-CreERT2* as described previously^{13,22,28,40–42}. Mice used in this study were age- and gender-matched littermates including both sexes. All mice were housed in the animal facility of Stanford University on a 12-h light/dark cycle with *ad libitum* access to water and normal chow except when otherwise indicated. All animal experiments and methods were approved by the Institutional Animal Care and Use Committee of Stanford University.

Physiological studies

We performed glucose physiology studies as previously reported¹³. Briefly, at the ages or times indicated in the text and figures, or after tamoxifen administration, we monitored body weight and blood glucose levels in *ad libitum* fed mice, or after 16 h overnight fasting, or 2 h after re-feeding ('2 h postprandial'). We drew blood from tail vein punctures or from the aorta when mice were killed, for measuring blood glucose levels using a Contour glucose meter (Bayer), or for measuring plasma insulin levels by mouse insulin ELISA kits (ALPCO Diagnostics).

Glucose tolerance tests were performed on mice after a 16 h overnight fast, and the blood glucose levels were determined immediately before (0) and 5, 10, 20, 30, 45, 60, 90 and 120 min after intraperitoneal injection of glucose (2 g kg⁻¹ body weight). For measurement of plasma insulin levels during these glucose tolerance tests, mice were injected with glucose at a dose of 3 g kg⁻¹ body weight, and we collected tail vein blood at 0, 15 and 45 min after

glucose injection followed by mouse insulin ELISA (ALPCO diagnostics). For insulin tolerance tests, mice were fasted for 6 h then received intraperitoneal injection of 0.75 units kg^{-1} of body weight of insulin (Sigma); glucose levels at 0, 10, 20, 30, 40, 60 and 90 min after injection were then measured.

For detecting *in vivo* β -cell proliferation by BrdU incorporation, mice were either fed with water containing BrdU (1 mg ml^{-1}) or intraperitoneally injected with BrdU/PBS solution (50 mg kg^{-1} body weight) for specified schedules as follows: $\beta\text{PdgfraKO}$ mice, 2 days of intraperitoneal injection of BrdU/PBS; $\beta\text{PdgfraKO}$ mice with STZ treatment, 1 week of BrdU water feeding; $\beta\text{PDGFR}\alpha\text{Tg}$ or $\beta\text{Ezh2KO-R}\alpha\text{Tg}$ mouse studies, 6–7 days of BrdU water feeding; RbTriKO mice, 4 days of BrdU water feeding. After BrdU chase, mouse pancreases were collected and subjected to immunohistology followed by morphometric analyses described later.

STZ-induced diabetes

For measuring islet *Pdgfra* and *Pdgfrb* induction during STZ-induced diabetes, male wild-type C57BL/6 mice, aged 8 weeks, were injected intraperitoneally with 100 mg kg^{-1} body weight of freshly dissolved streptozotocin (STZ, Sigma) in 0.1 mol l^{-1} sodium citrate (pH 4.5) or with the same volume of sodium citrate (Vehicle). Six days later, islets or pancreases were recovered from the mice for gene expression analyses by real-time RT-PCR or immunohistology studies. To test the roles of *Pdgfra* in islet regeneration in STZ diabetes, $\beta\text{PdgfraKO}$ or control mice (including $\beta\text{PdgfraH}\alpha\text{t}$), aged 7–8 weeks, were treated with a single intraperitoneal STZ injection at a dose of 150 mg kg^{-1} body weight. After injection, blood glucose values were measured on day 3 and day 6 in the first week, and thereafter once per week. After 2 weeks of STZ treatment, subsets of $\beta\text{PdgfraKO}$ and control littermates were fed with BrdU-containing drinking water (1 mg ml^{-1}) for 1 week for BrdU incorporation analyses. Three days, 21 days and 58 days after STZ treatment, subsets of mice were killed, and blood samples and pancreases were harvested for measurement of plasma insulin levels by ELISA, and for pancreatic β -cell proliferation and mass quantification following immuno-staining of insulin and BrdU.

Islet isolation and culture studies

Mouse pancreatic islets were purified as described previously¹³ and used immediately for assays described in the text. To study *Ezh2* induction in cultured islets exposed to mitogens, freshly isolated islets from 2–3-week old, and 7–12-month old C57BL/6 mice were recovered and equilibrated overnight at $37\text{ }^{\circ}\text{C}$, 5% CO_2 in RPMI-1640 medium supplemented with 0.5% fetal bovine serum (FBS), 0.2% bovine albumin (BSA) and 2% penicillin/streptomycin. Afterwards, islets were distributed into microplates or dishes, and treated with recombinant human PDGF-AA, prolactin or insulin (Sigma), with or without addition of pharmacological inhibitors, including Sunitinib (Selleck), Vargatef (Selleck), U0126 (Sigma), LY294002 (Sigma) or U-73122 (Sigma) at the indicated concentrations. Two days later, islets were harvested for RNA or protein extraction for real-time RT-PCR or western blot analyses. To detect phosphorylation of $\text{Pdgfr-}\alpha$ by western blot, islets were exposed to PDGF-AA (with or without indicated pharmacological inhibitors) for 90 min before islet harvesting and protein extraction. To detect *in vitro* islet β -cell proliferation stimulated by PDGF-AA, a final concentration of $50\text{ }\mu\text{M}$ BrdU was introduced into each culture dish 24 h before harvest, followed by standard histological processing. Immunostaining of BrdU and insulin was performed on processed islets, and β -cell proliferation rate was determined by quantifying percentage of insulin⁺ cells with BrdU⁺. Culture experiments of human islets were performed using a similar procedure described later.

β-cell purification by FACS

Freshly purified islets from MIP-GFP mice at 2 weeks or 5 months of age were pooled, and dispersed into a suspension of single cells by incubation with 0.05% trypsin/0.53mM EDTA solution at 37 °C for 10 min as described previously^{37,43}. After dissociation, cells were washed three times in PBS containing 2% FBS, and re-suspended in FACS buffer (HBSS/0.2% BSA/1% HEPES) containing propidium iodide (to exclude dead cells). FACS was performed on a FACS AriaII (BD Biosciences), and MIP-GFP⁺ β-cells with >95% purity were harvested and immediately lysed for RNA purification and real-time RT-PCR analysis.

Real-time RT-PCR and western blot analysis

Total RNA from freshly isolated or cultured islets after indicated treatments were purified using the Absolutely RNA miniprep purification kit (Stratagene) according to the manufacturer's instructions. RNA concentration was measured with a RiboGreen RNA quantification assay (Invitrogen). One-step quantitative RT-PCR was performed and analysed using an ABI Prism 7300 detection system (Applied Biosystems) with TaqMan one-step RT-PCR Master Mix Reagents and appropriate amounts (10–100 ng) of islet total RNAs as the template. We calculated the ratio of mRNA for the gene of interest to the amount of internal control mRNA of peptidylprolyl isomerase A (cyclophilin A, PPIA), and then normalized the ratio for each gene to its median. Primer and probe sequences are listed in Supplementary Tables 1 and 2.

For western blots, total islet protein was prepared from freshly isolated or cultured islets after indicated treatments as previously described¹³. Equal amounts of protein were resolved on SDS-PAGE and transferred to polyvinylidene fluoride membranes (Amersham Pharmacia) for immunoblotting with specific antibodies, including rabbit polyclonal anti-human Ezh2 (1:1,000, Millipore), rabbit polyclonal anti-phospho-PDGFR-α (Tyr 720) (1:1,000, Santa Cruz Biotechnologies), rabbit monoclonal anti-PDGFR-α (1:1,000, Cell Signaling), mouse monoclonal anti-β-actin (1:4,000, Sigma), rabbit monoclonal anti-Akt (1:1,500, Cell Signaling), rabbit monoclonal anti-phospho-Ark (Ser 473) (1:1,000, Cell Signaling), rabbit monoclonal anti-p44/42 MAPK (Erk1/2) (1:1,500, Cell Signaling), rabbit monoclonal anti-phospho-p44/42MAPK (Erk1/2) (Thr 202/Tyr 204) (1:1,000, Cell Signaling), rabbit monoclonal anti-PLCγ1 (1:1,000, Cell Signaling) and rabbit monoclonal anti-phospho-PLCγ1 (Tyr 783) (1:1,000, Cell Signaling). Signal was visualized using ECL detection (Amersham Pharmacia) on Kodak film after further incubation with HRP-conjugated secondary antibodies. For phospho-PDGFR-α (Tyr 720) and PDGFR-α detection, signal was amplified by incubating the membrane with biotinylated anti-rabbit-IgG (1:2,000), followed with an incubation with HRP-avidin (1:2,000, Vector Laboratories) and then ECL visualization.

Histology, immunofluorescence and immunohistochemistry

We performed standard histological paraformaldehyde fixation, paraffin-embedding and immunostaining protocol as previously described¹³. Briefly, immunohistochemical analysis was performed on 5-μm-thick sections of pancreatic tissues or islet sections after antigen retrieval (DAKO) and using the following primary antibodies: guinea pig anti-insulin (1:400, Sigma), mouse anti-glucagon (1:200, Sigma), rabbit anti-somatostatin (1:200, Dakocytomation), rabbit anti-pancreatic polypeptide (1:200, Dakocytomation), rabbit anti-Ezh2 (1:100, Epigentek), rabbit anti-PDGFR-α (1:50, Novus Biologicals), sheep anti-PDGFR-β (1:100, Sigma), rabbit anti-phospho-PDGFR-α (Tyr 720) (1:50, Santa Cruz Biotechnologies), rabbit anti-phospho-p44/42 MAPK (Erk1/2) (Thr 202/Tyr 204) (1:50, Cell Signaling), rabbit anti-phospho-Rb (Ser 780) (1:100, Cell Signaling), rabbit anti-cyclin D1 (1:50, Cell Signaling), rabbit anti-cyclin D2 (1:50, Cell Signaling), mouse anti-PDX1 (1:50;

gift from H. Edlund), mouse monoclonal anti-Ki67 (1:100, Novocastra) and mouse monoclonal anti-BrdU (1:100, Sigma). We detected immune complexes with secondary antibodies conjugated with either Cy3, fluorescein isothiocyanate (Jackson ImmunoResearch) or horseradish peroxidase (Vector Laboratories). After staining, images were directly collected on an AxioM1 microscope equipped with a CCD digital camera (Carl Zeiss), or on a Leica SP2 inverted confocal microscope.

For measuring pancreatic β -cell mass or BrdU by morphometry, three to six mice for each group were analysed. At least five sections separated by more than 300 μ m (pancreases) or 50 μ m (cultured islets) were immunostained and assessed for each sample. Images were analysed with an ImagePro program by observers blinded to genotype, and pancreatic β -cell mass and percentage of BrdU⁺ β -cells were calculated as described previously¹³.

Pancreatic islet ChIP analysis

ChIPs were performed using a chromatin immunoprecipitation kit from Millipore as described previously¹³. Briefly, freshly isolated mouse pancreatic islets or cultured human islets after the indicated treatments were pooled, and fixed with 2% formaldehyde for cross-linking for 20 min at room temperature (25 °C). After washing, islets were dissolved in SDS lysis buffer containing a proteinase inhibitor cocktail followed by sonication to shear the chromatin. Precleared chromatin from 150 to 300 islets was used for each ChIP sample with incubation of 1 to 5 μ g of the appropriate antibodies at 4 °C overnight. The antibodies used in the ChIP assays included mouse anti-E2F1 (Millipore), rabbit anti-E2F4 (Santa Cruz Biotechnologies), rabbit or mouse polyclonal IgG (Millipore). After incubation, the immunoprecipitated chromatin DNA was harvested, cross-link reversed, and purified. After measurement of DNA concentration by PicoGreen DNA assay (Invitrogen), equivalent amounts of chromatin DNA from every sample was quantified by real-time qPCR in ABI Prism 7300 detection system (Applied Biosystems). The sequences of the PCR primers and probes are listed in Supplementary Table 3.

Human pancreas and islet studies

Human pancreases or islets from organ donors were procured by arrangement with the National Disease Resource Interchange and the University of Alabama, Birmingham with appropriate consent. Institutional review board approval for research use of tissue was obtained from Stanford University School of Medicine. For pancreas studies, fresh human pancreases were processed in our laboratory into paraffin sections using standard histological protocols. Four pancreases from juvenile donors (1, 2, 3, 7 years old) and four pancreases from adult donors (22, 34, 55, 56 years old) were used for immunohistology studies here. Similar immunohistology methods and antibodies as those used for mouse pancreas tissues were used to detect PDGFR- α , phospho-ERK1/2 (pERK1/2), phospho-RB (Ser 780) (pRB(Ser780)), EZH2 and insulin on human pancreas sections. Experiments were repeated on multiple pancreas sections for every donor pancreas and similar results were obtained.

For islet studies, human islets were isolated either at Pittsburgh University Medical Center (R.B.), or the University of Alabama, Birmingham (S. Bryant and A. Thompson). Eight independent human islet preparations from juvenile donors at the age of 8 months, 1.0, 1.5, 2, 3, 4, 5 and 6 years, and three adult islet preparations from donors at the age of 39, 48 and 56 years were used in this study. After isolation, fresh islets were shipped to our laboratory, and were hand-picked and transferred to RPMI-1640 medium supplemented with 0.5% FBS, 0.2% BSA and 2% penicillin/streptomycin for an overnight recovery before experiments. Similar to the experiments performed with mouse islets, equilibrated human islets were exposed to recombinant human PDGF-AA, without or with addition of pharmacological

inhibitors, including Sunitinib (Selleck) and U0126 (Sigma) for 2 days, followed by islet RNA purification for real-time RT-PCR analyses of gene expression. To detect *in vitro* human islet β -cell proliferation stimulated by PDGF-AA, human islets were exposed to 50 μ M BrdU for 24 h. Afterwards, human islets were processed in a similar way as in mouse islet studies for immunostaining of insulin, glucagon, PDX1 and BrdU. β -cell proliferation rate was determined by quantifying percentage of insulin⁺ cells with BrdU⁺. More than 50 islets per group were counted for determining cultured human islet β -cell proliferation rate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Age-dependent attenuation of Pdgfr- α limits β -cell *Ezh2* expression and proliferation in neonatal and juvenile mice

a, Pdgfr- α and insulin immunostaining on wild-type mouse pancreas sections at indicated ages. DAPI, 4',6-diamidino-2-phenylindole. Scale bar, 25 μ m. **b**, **c**, mRNA levels of indicated genes in FACS-purified β -cells (**b**) or wild-type islets (**c**) at specified ages. $n = 3$ –5 experiments per group or time point. * $P < 0.05$, ** $P < 0.01$ compared to samples from 2 weeks (**b**) or 15 days (**c**). *Insr* refers to mRNA encoding insulin receptor. **d**–**g**, Relative mRNA levels of *Pdgfra* (**d**) and *Ezh2* (**e**) in isolated islets, and percentage β -cell BrdU incorporation (**f**) and pancreatic β -cell mass (**g**) of β PdgfraKO and control mice at specified ages. $n = 3$ –5 independent islet preparations or mice per group. * $P < 0.05$, ** $P < 0.01$ for the comparison as indicated. Error bars denote s.e.m.

Figure 2. *Pdgfra* loss impairs β -cell regeneration in STZ-induced diabetes

a, b, Immunostaining of Pdgfr- α and insulin on pancreas sections (**a**), and relative mRNA levels of *Pdgfra* and *Pdgfrb* in islets (**b**) from 8-week-old wild-type mice 1 week after vehicle or STZ (100 mg kg⁻¹ body weight) treatment. $n = 3$ –5 mice per group. **a**, Yellow arrows mark Pdgfr- α induction in a subset of insulin⁺ cells. **c**, Representative images showing immunostaining for insulin, Ezh2 and BrdU on pancreatic sections from indicated 6–7-week-old mice 3 weeks after STZ (150 mg kg⁻¹ body weight) treatment. **d**, Percentage of BrdU⁺ insulin⁺ cells (β -cells) or BrdU⁺ acinar cells 3 weeks after STZ challenge. $n = 3$ or 6 mice per group. **e, f**, Pancreatic β -cell mass (**e**) and blood glucose (**f**) levels during *ad libitum* feeding after STZ treatment. $n = 3$ –8 mice (**e**) or 18–21 mice (**f**) per time point per group. * $P < 0.05$, ** $P < 0.01$ for the comparison as indicated or versus control. Scale bars, 25 μ m. Error bars denote s.e.m.

Figure 3. Activated PDGFR- α delays age-dependent Ezh2 loss and replication failure in pancreatic β -cells

a, Relative mRNA levels of human *PDGFRA* and mouse *Pdgfra* in islets from littermate control and β PDGFR α Tg mice at 3 months of age. $n = 3-7$ mice per group. **b-d**, Pancreatic β -cell mass (**b**), and β -cell proliferation, assessed by Ki67 expression (**c**) or BrdU incorporation (**d**) in β Pdgfra.KO and control mice at indicated ages. $n = 3-5$ mice per group. **e**, **f**, Relative mRNA levels of *Ezh2* (**e**) in islets, and immunostaining (**f**) for Ezh2, BrdU and insulin on pancreatic sections from control and β PDGFR α Tg mice at indicated ages. $n = 3-7$ mice per group. Scale bars, 25 μ m. **g**, Postprandial blood glucose levels in mice at indicated ages $n = 14-24$ mice per group per time point. **h**, Glucose tolerance assessed in 14-month-old control and β PDGFR α Tg mice. $n = 6$ or 10 mice per group. * $P < 0.05$, ** $P < 0.01$ in comparisons indicated. Error bars denote s.e.m.

Figure 4. PDGFR- α promotes β -cell expansion through *Ezh2*

a, b, mRNA levels for human *PDGFRA* (**a**) and mouse *Ezh2* (**b**) in islets from mice with indicated genotypes at 3–4 months of age. $n = 3$ –5 mice per group. **c–f**, β -cell BrdU incorporation (**c**), β -cell mass (**d**), postprandial plasma insulin (**e**) and blood glucose (**f**) levels in 3–4-month-old mice with indicated genotypes. Each dot in **f** represents a measurement from an individual mouse. $n = 4$ –5 mice for **c** and **d**, and $n = 5$ –18 mice for **e** and **f** per genotype. * $P < 0.05$, ** $P < 0.01$. NS, not significant in comparisons indicated. Error bars denote s.e.m.

Figure 5. Pdgfr signalling governs Erk and Rb/E2f regulation of *Ezh2* in islet β -cells

a, Western blot analysis of total and phosphorylated Erk1/2, Akt and PLC γ in islet proteins from 3–4-month-old β PDGFR α Tg and littermate control mice. Similar results were obtained from multiple independent experiments. **b**, Relative phosphorylated protein level compared to total protein was quantified by densitometry. **c**, Immunostaining for phospho-Erk1/2 (pErk1/2), phospho-Rb-Ser 780 (p-Rb(Ser780)) and insulin in 3-month-old littermate control and β PDGFR α Tg pancreatic sections. Scale bar, 25 μ m. **d**, Percentage of phospho-Rb-Ser 780⁺ insulin⁺ cells from the indicated mice quantified by morphometry. *n* = 4 mice per group. **e**, Cyclin D1 (*Ccnd1*) or cyclin D2 (*Ccnd2*) mRNA levels in 3-month-old control and β PDGFR α Tg islets. *n* = 4 or 6 mice per group. **f**, **g**, ChIP analyses of the *Ezh2* and β -actin (*Actb*) loci with antibodies to E2f1 (**f**) and E2f4 (**g**) using the indicated amplicons (see Supplementary Information) in 3–4-month-old littermate control and β PDGFR α Tg islets. *n* = 3–4 independent experiments per antibody with independent islet samples. **P* < 0.05, ***P* < 0.01 for control versus β PDGFR α Tg. Error bars denote s.e.m.

Figure 6. PDGFR- α regulates human β -cell *EZH2* expression and proliferation

a, b, Representative images showing immunostaining for PDGFR- α (**a**), phospho-ERK1/2 (pERK1/2), phospho-RB-Ser 780 (pRB(Ser780)), *EZH2* (**b**) and insulin (**a** and **b**) on pancreatic sections from juvenile and adult human subjects. PDGFR- α was detected in juvenile β -cells (arrows) but not in adult β -cells (arrowheads). Scale bars, 25 μ m.

c-f, Assessment of effects on human juvenile or adult islets after exposure to PDGF-AA (50 ng ml⁻¹) for 2 days, with or without Sunitinib (2 μ M) or U0126 (10 μ M) co-treatment. **c**, Islet *EZH2* mRNA levels after the indicated treatments. $n = 3-5$ independent experiments. **d**, *EZH2* locus ChIP analysis with anti-E2F1 antibody or IgG in human juvenile islets. $n = 3-4$

for E2F1, $n = 2$ for IgG. *e, f*, Human islet β -cell proliferation changes after the indicated treatments. Average percentage of BrdU⁺ insulin⁺ cells (**e**) was quantified by morphometry from sectioned islets immunostained (*f*) for insulin (green), glucagon (white) and BrdU (red). $n = 3-6$ independent experiments. Scale bar, 25 μ m.

g, Illustration summarizing how Pdgfr- α signalling regulates β -cell *Ezh2* and proliferation by activating Erk/Rb/E2f pathways sensitive to the indicated inhibitors. * $P < 0.05$, ** $P < 0.01$ as indicated. Error bars denote s.e.m.