


Placental growth factor in beta cells plays an essential role in gestational beta-cell growth

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ABSTRACT

Objective Pancreatic beta cells proliferate in response to metabolic requirements during pregnancy, while failure of this response may cause gestational diabetes. A member of the vascular endothelial growth factor family, placental growth factor (PIGF), typically plays a role in metabolic disorder and pathological circumstance. The expression and function of PIGF in the endocrine pancreas have not been reported and are addressed in the current study.

Research design and methods PIGF levels in beta cells were determined by immunostaining or ELISA in purified beta cells in non-pregnant and pregnant adult mice. An adeno-associated virus (AAV) serotype 8 carrying a shRNA for PIGF under the control of a rat insulin promoter (AAV–rat insulin promoter (RIP)–short hairpin small interfering RNA for PIGF (shPIGF)) was prepared and infused into mouse pancreas through the pancreatic duct to specifically knock down PIGF in beta cells, and its effects on beta-cell growth were determined by beta-cell proliferation, beta-cell mass and insulin release. A macrophage-depleting reagent, clodronate, was coapplied into AAV-treated mice to study crosstalk between beta cells and macrophages.

Results PIGF is exclusively produced by beta cells in the adult mouse pancreas. Moreover, PIGF expression in beta cells was significantly increased during pregnancy. Intraductal infusion of AAV–RIP–shPIGF specifically knocked down PIGF in beta cells, resulting in compromised beta-cell proliferation, reduced growth in beta-cell mass and impaired glucose tolerance during pregnancy. Mechanistically, PIGF depletion in beta cells reduced islet infiltration of trophic macrophages, which appeared to be essential for gestational beta-cell growth.

Conclusions Our study suggests that increased expression of PIGF in beta cells may trigger gestational beta-cell growth through recruited macrophages.

INTRODUCTION

During pregnancy, the increase in maternal blood glucose requires the pancreas to produce more insulin to keep normal glucose homeostasis,¹ and failure of this compensation may cause gestational diabetes.² Although the high blood sugar in gestational diabetes usually returns to normal after pregnancy, previous studies have shown that gestational diabetes is a predisposing factor for

Significance of this study

What is already known about this subject?

- The expression and function of placental growth factor (PIGF) in the endocrine pancreas have not been studied before.

What are the new findings?

- PIGF is exclusively expressed by beta cells in adult pancreas.
- Increased beta cell-derived PIGF promotes gestational beta-cell growth through macrophages.

How might these results change the focus of research or clinical practice?

- The contribution of insufficient beta cell-derived PIGF to the development of gestational diabetes deserves further investigation in the clinic.

type 2 diabetes, which occurs later in the life of pregnant women.³ Hence, understanding the etiology and pathogenesis of gestational diabetes is extremely important, given that this disease affects up to 10% of pregnant women.⁴

Studies on pregnant women and rodents have suggested that the gestational increases in insulin production and secretion by beta cells may be largely attributable to beta-cell growth during this period.^{5–10} Indeed, numerous studies have shown that gestational increase in beta-cell mass primarily stems from beta-cell proliferation.^{11–14} Failure to adequately increase beta-cell mass may cause glucose intolerance or even gestational diabetes. To date, the molecular mechanisms underlying gestational beta-cell growth are not fully understood.^{5–10}

The intimate relationship between beta cells and endothelial cells starts during embryonic pancreatic organogenesis¹⁵ and plays a critical role in both endocrine pancreas development and beta-cell function in adults.¹⁶ This interaction is mainly mediated by vascular



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endothelial growth factor A (VEGF-A), the best studied ligand from the vascular endothelial growth factor (VEGF) family.¹⁷ We have previously shown that VEGF-A plays a role in the control of beta-cell mass in response to glucose alterations in the circulation,¹⁸ while the two major sources of VEGF-A in pancreas are beta cells and duct cells, which differentially regulate VEGF-A release.¹⁹ These earlier studies contributed to our understanding of the role of VEGF-A in beta-cell biology. However, VEGF family members other than VEGF-A biology have been minimally studied in beta-cell biology.

Placental growth factor (PIGF) is another member of the VEGF family and is thought to play a role in altered metabolic states or under some pathological circumstance.²⁰ The expression and function of PIGF in the endocrine pancreas have not been examined. Here, we investigated PIGF expression in beta cells, as well as its role in gestational beta-cell growth.

MATERIALS AND METHODS

Production of AAV8 expressing shPIGF under a rat insulin promoter (RIP)

A PIGF shRNA sequence (GCTGTTCACCTTGCTTCTTAGTCGAGTAAGAAGCAAGTGAACAGC) or a scramble (GCTGAGTACTTCGAAATGTCGTCGAGGACATTTCCGAGTACTCAGCG) was designed using Block-IT RNAi designer and was cloned into pAAV-mcherry-flex-dTA (dTA) (Addgene, No 58536, Cambridge, Massachusetts, USA) by restriction sites BsrGI and SalI. A RIP described before²¹ was swapped into the vector by restriction sites FseI and XbaI. Adeno-associated virus (AAV) serotype 8 vectors were generated by cotransfection with the shPIGF or the scrambled plasmid, a packaging plasmid carrying rep and cap from the AAV serotype 8, and a helper plasmid carrying the adenovirus helper in human embryonic kidney 293 cells, as described before.²² AAV viruses were purified by polyethylene glycol/aqueous two-phase partitioning and then stored at -80°C for later use. Titration of viral vectors was determined by an AAVpro Titration kit (TaKaRa Bio, Mountain View, California, USA). Recombinant mouse PIGF was purchased from Abcam (Ab207150, Cambridge, Massachusetts, USA) and was delivered to mice via pancreatic duct at $20\mu\text{g}/\text{mouse}$. The control mice received saline. Transfection of a beta-cell line Min6 (American Type Culture Collection, Rockville, Maryland, USA) was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, California, USA). Min6 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplied with 10% fetal bovine serum (Invitrogen), $2\text{mmol}/\text{L}$ glutamine at 37°C , 95% air/5% CO_2 .

Mouse manipulations

The BAC transgenic elastase promoter CreERT reporter (Ela-CreERT) mouse was described before.¹⁹ C57BL/6, Rosa26CAGTomato (Tomato)¹⁸ and INS1^{cre} knock-in²³ mice were all purchased from the Jackson Lab (Bar

Harbor, Maine, USA). Tamoxifen induction of Tomato expression in acinar cells in Ela-CreERT; Tomato mice has been described before.¹⁹ INS1^{cre} knock-in mice were bred with Tomato mice to generate INS1^{cre}; Tomato mice for purification of beta-cells based on red fluorescence. Fasting blood glucose monitoring and intraperitoneal glucose tolerance testing were performed as described before.²¹ Pancreatic intraductal virus infusion was performed as described.²⁴ Injection of $200\mu\text{L}$ clodronate and control liposome (clodronate liposomes, Netherlands) from the tail vein of the mice was performed twice per week, starting 1 day before time breeding until the end of the experiment, as previously described.²¹

RNA isolation and quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, California, USA) and then quantified with the Nanodrop 1000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), followed by cDNA synthesis (Qiagen) and RT-qPCR. Primers for CycloA (QT00247709) and PIGF (QT00103222) were both purchased from Qiagen. Relative values of mRNA levels were obtained by sequential normalization against the housekeeping gene cyclophilin A and the experimental control.

PIGF ELISA

PIGF levels were determined with a PIGF ELISA kit (R&D Systems, Los Angeles, California, USA). All reagents, standard dilutions and samples were prepared as directed in the product insert. First, $100\mu\text{L}$ of Assay Diluent was added to each well, followed by $100\mu\text{L}$ of standard, control, or sample to each well. The microplate was then covered with a plate sealer and incubated at room temperature for 2 hours. Each well was aspirated and washed three times, after which $200\mu\text{L}$ of conjugate was added to each well. The microplate was then covered with a new plate sealer and incubated at room temperature for 1 hour before another four times of aspiration and wash. Finally, $200\mu\text{L}$ substrate solution was added to each well, and the microplate was incubated at room temperature for 30 min before $50\mu\text{L}$ of stop solution was added to each well. The microplate was read at 450nm within 30 min. The wavelength correction was set to 540 or 570nm .

Pancreatic digestion and FACS

Pancreatic duct perfusion, pancreas digestion, islet isolation, single islet cell preparation, fluorescence-activated cell sorting (FACS) and confirmation of the sorted cell purity were performed as described before.¹⁹

Immunohistochemistry, quantification of proliferating beta cells and beta-cell mass

All pancreas samples were fixed and cryo-protected in 30% sucrose overnight before freezing, as described before.²⁵ Fast red staining was performed with the chromogen system (Dako, Carpinteria, CA, USA). For fluorescent staining, mCherry was detected by direct

fluorescence. Primary antibodies used were: guinea pig polyclonal anti-insulin (Dako), goat polyclonal anti-PIGF (R&D Systems), rat polyclonal anti-Ki-67 (Dako) and rat anti-F4/80 (Invitrogen). Secondary antibodies were all purchased from Dako. Nuclear staining (Hoechst 33342 (HO)) was performed with Hoechst 33342 (Becton-Dickinson Biosciences, San Jose, California, USA). Quantification of beta-cell proliferation was based on the percentage of Ki-67+ cells in total beta cells, as previously described.²⁶ Beta-cell mass was analyzed as described before.²¹ Briefly, the mouse pancreata were trimmed of all non-pancreatic tissue, weighed, fixed, cryoprotected in 30% sucrose overnight before freezing in a way to allow longitudinal sections from tail to head of the pancreas to be obtained. Sections at 150 µm intervals from whole pancreas were immunostained for insulin. The beta-cell mass per pancreas was estimated as the product of the relative cross-sectional area of beta cells per total tissue and the weight of the pancreas.

Data analysis

For in vivo experiments, five mice were used for each group. All data were statistically analyzed by one-way analysis of variance with a Bonferroni correction, followed by Fisher's exact test. All error bars represent SD. Significance was presented as * when $p < 0.05$. No significance was presented as NS. P value and n value are indicated in the figure legends.

RESULTS

PIGF is exclusively produced by beta cells in the adult mouse pancreas

VEGF-A in beta cells plays an important role in the control of beta-cell development and function, while the role of PIGF in beta cells is unknown. Here, we found that in the mouse pancreas, PIGF+ cells were exclusively located in the islet (figure 1A). We compared PIGF transcripts among different FACS-purified pancreatic cell types (beta cells (red fluorescent cells from islets of *INS1^{cre}*; Tomato mice), non-beta-cells (non-red fluorescent cells from islets of *INS1^{cre}*; Tomato mice), duct cells (Dolichos biflorus agglutinin (DBA)+ cells from C57BL/6 mice), endothelial cells (CD31+ cells from C57BL/6 mice) and acinar cells (Tomato+ cells from tamoxifen-treated *El-CreERT*; Tomato mice)) (figure 1B). We found that beta cells were the only pancreatic cell type that expressed significant PIGF (figure 1C).

PIGF in beta cells upregulates during pregnancy

It is well known that pancreatic beta cells proliferate in response to metabolic needs during pregnancy, while failure of this response may lead to gestational diabetes. Since beta cells produce both VEGF-A and PIGF, we then examined expression of both VEGF-A and PIGF in beta cells in pregnant *INS1^{cre}*; Tomato mice. Interestingly, we found that VEGF-A levels in beta cells remain unchanged during pregnancy but significantly increased at gestational day 16 (G16, the peak of beta-cell proliferation in

pregnant mice),¹⁴ compared with non-pregnant (NP) litter mates, by RT-qPCR (figure 1D) and by ELISA (figure 1E). PIGF and insulin coimmunostaining was done on pancreas from NP and G16 mice, and both showed beta cell-specific PIGF expression (figure 1F). The PIGF signals appeared to be higher in G16, compared with NP (figure 1F). These data suggest that PIGF may play a role in gestational beta-cell growth. VEGFR1 is the unique receptor for PIGF, and VEGFR1 is expressed in both macrophages and endothelial cells, but not in any endocrine cells.^{19 27-30} Thus, we examined the levels of VEGFR1 in sorted islet macrophages and endothelial cells. We did not detect alteration in VEGFR1 levels on these cells (online supplementary figure 1), suggesting that VEGFR1 expression level is not a cause for increased beta cell-derived PIGF during pregnancy.

Beta cell-specific knockdown of PIGF

In order to evaluate the role of PIGF during gestational beta-cell growth, we used AAV serotype 8 carrying a short hairpin small interfering RNA for PIGF (shPIGF) under the control of a RIP to specifically deplete PIGF in beta cells (AAV-RIP-shPIGF). An AAV carried a scrambled sequence under RIP (AAV-RIP-scrambled) as controls. An mCherry reporter was also included in the AAV backbone to allow the transduced cells to be visualized by red fluorescence (figure 2A). The mouse islet endothelial cell line MS1 (a non-beta-cell line used as a control) and mouse insulinoma cell line Min6 were both transduced by AAV-RIP-shPIGF (figure 2B). PIGF mRNA was significantly knocked down in Min6 cells but was unaltered in MS1 cells, confirming the specificity of RIP and efficient knockdown of shPIGF in AAV-RIP-shPIGF (figure 2C). Moreover, PIGF protein in the cellular extracts and in conditioned media from AAV-RIP-shPIGF-transduced Min6 cells was reduced by more than 70% (figure 2D).

Depletion of PIGF in beta cells impairs the glucose response during pregnancy

The AAV-RIP-shPIGF or AAV-RIP-scrambled virus was infused into mouse pancreas from the pancreatic duct to specifically knock down PIGF in beta cells in 10-week-old female C57BL/6 mice as described.^{21 25 31} Three weeks later, the incision on these mice was healed, and some mice were analyzed, showing no difference in beta-cell proliferation and beta-cell mass between two experimental groups (online supplementary figure 2). Other mice were time-mated with male C57BL/6 mice. The pregnant mice were sacrificed at G16, the peak of beta-cell proliferation as reported by us¹⁴ and by others⁵⁻¹⁰ (figure 3A). At G16, we detected red fluorescent cells only in beta cells (about 80% of beta cells were red) from the mice that had received either AAV-RIP-shPIGF or AAV-RIP-scrambled, suggesting that these viruses only transduced beta cells, which again confirmed the specificity of the RIP in the viral structure (figure 3B-C). Moreover, PIGF expression was greatly attenuated in beta cells from AAV-RIP-shPIGF-treated mice, compared

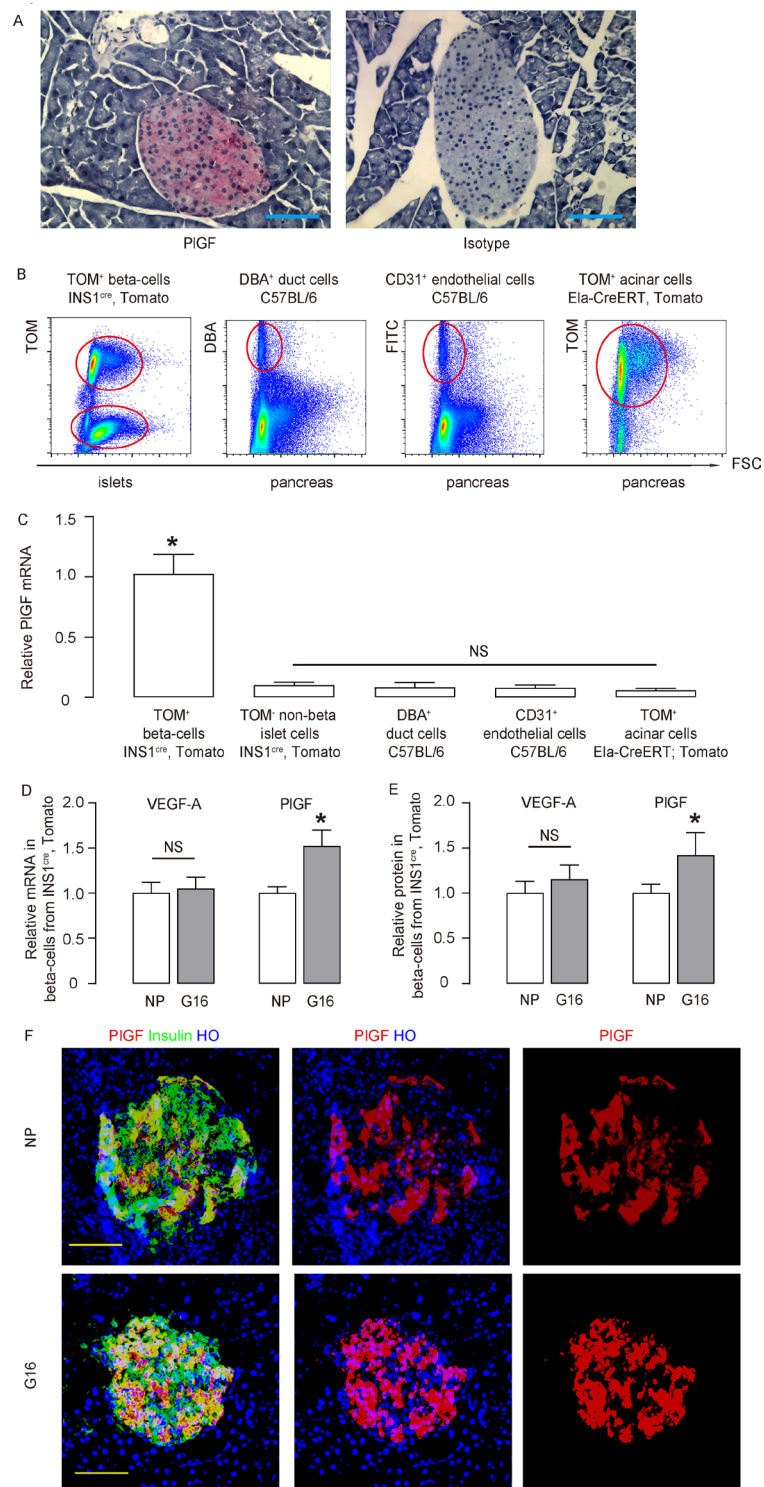


Figure 1 PIGF expresses in the adult beta cells and upregulates during pregnancy. (A) Immunostaining for PIGF (fast red) in normal mouse pancreas, compared with the isotype antibody control. (B) Representative FACS charts to show purification of beta cells (red fluorescent cells from islets of INS1^{cre}, tomato mice), non-beta cells (non-red fluorescent cells from islets of INS1^{cre}, tomato mice), duct cells (DBA+ cells from C57BL/6 mice), endothelial cells (CD31+ cells from C57BL/6 mice) and acinar cells (tomato+ cells from tamoxifen-administrated Ela-CreERT, tomato mice). (C) RT-qPCR for PIGF mRNA levels among different FACS-purified pancreatic cell types. (D,E) VEGF-A and PIGF mRNA and protein levels were determined in purified beta cells from INS1^{cre}, tomato mice, by RT-qPCR (D) and by ELISA (E), respectively, in the NP mice and litter mate pregnant mice at G16. (F) Representative immunostaining for PIGF and insulin in the NP and G16 mouse pancreas. **p*<0.05. *n*=5. Scale bars are 50 μ m. DBA, dolichos biflorus agglutinin; Ela-CreERT, elastase promoter CreERT reporter; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; G16, gestational day 16; HO, Hoechst 33342; mRNA, messenger RNA; NP, non-pregnant; NS, non-significant; PIGF, placental growth factor; RT-qPCR, quantitative reverse transcription PCR; TOM, tomato; VEGF-A, vascular endothelial growth factor A.

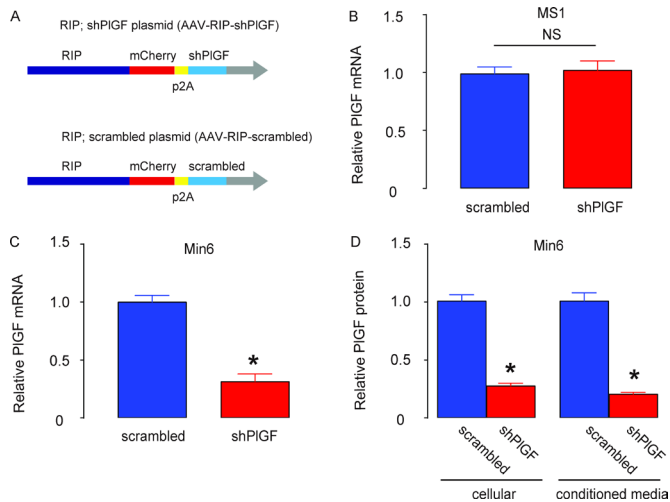


Figure 2 Beta cell-specific knockdown of PIGF. (A) In order to evaluate the role of PIGF during gestational beta-cell growth, we used AAV serotype 8 carrying an shPIGF under the control of an RIP to specifically inhibit PIGF production in beta cells (AAV-RIP-shPIGF). A control AAV carried a scrambled sequence under RIP (AAV-RIP-scrambled). An mCherry reporter was also included in the AAV backbone to allow transduced cells to be visualized by red fluorescence. (B,C) RT-qPCR for PIGF in MS1 (B) and MIN6 (C) cells transduced with AAV-RIP-shPIGF or AAV-RIP-scrambled. (D) ELISA for PIGF in cellular compartment or conditioned media in AAV-RIP-shPIGF-transduced MIN6 cells. * $p < 0.05$. $n = 5$. AAV, adeno-associated virus; NS, non-significant; PIGF, placental growth factor; RIP, rat insulin promoter; RT-qPCR, quantitative reverse transcription PCR; shPIGF, short hairpin small interfering RNA for PIGF.

with beta cells from AAV-RIP-scrambled-treated mice (figure 3D). Quantification of PIGF protein in isolated mouse islets showed more than 50% reduction in PIGF (figure 3E), suggesting successful knockdown of PIGF in beta cells in AAV-RIP-shPIGF-treated mice. At G16, although the fasting glycemia remained unaltered in AAV-RIP-shPIGF-treated mice (figure 3F), these mice exhibited compromised glucose response (figure 3G) and reduced serum insulin (online supplementary figure 3A), compared with AAV-RIP-scrambled-treated mice. Moreover, insulin tolerance test (ITT) did not show difference among the two groups of mice (online supplementary figure 3B). Therefore, beta cell-specific knockdown of PIGF leads to impairment of glucose response during pregnancy, likely through compromised beta-cell growth rather than through reduced insulin release.

Beta cell-specific knockdown of PIGF inhibits beta-cell proliferation, resulting in attenuated gestational beta-cell growth

Next, we explored the mechanisms underlying the impairment of the glucose response in AAV-RIP-shPIGF-treated pregnant mice. Beta-cell proliferation was determined by Ki-67 positivity in beta cells, and the outcome of the alteration in beta-cell proliferation was assessed by beta-cell mass at G16. We detected a significant reduction in

beta-cell proliferation in AAV-RIP-shPIGF-treated mice, compared with AAV-RIP-scrambled-treated mice, as shown by representative images (figure 4A) and by quantification (figure 4B). Moreover, the increases in beta-cell mass were attenuated in AAV-RIP-shPIGF-treated mice, compared with AAV-RIP-scrambled-treated mice (figure 4C). Thus, beta cell-specific knockdown of PIGF inhibits beta-cell proliferation, resulting in attenuated gestational beta-cell growth.

PIGF promotes gestational beta-cell proliferation through recruitment of macrophages

Recently, we and others have shown a role of macrophages in beta-cell proliferation and regeneration.^{16 21 32-34} As mentioned earlier, PIGF was a potent ligand for VEGFR1, which is expressed by macrophages.³⁵⁻³⁷ Thus, we hypothesized that the effects of beta cell-derived PIGF on beta-cell proliferation may be mediated by macrophages. To prove it, we examined the recruitment of macrophages to the mouse islets in virus-treated pregnant mice at G16. F4/80 is a pan-macrophage marker. While very few F4/80+ macrophages were detected in the NP mouse pancreas, many more macrophages were found inside and close to the islets in pregnant mice that had received AAV-RIP-scrambled, while this increase in macrophage infiltration into the islet region in pregnant mice was greatly reduced in AAV-RIP-shPIGF-treated mice (figure 5A,B). To prove that this increase in macrophages by PIGF in beta cells contributes to gestational beta-cell growth, we used a macrophage-depleting reagent, clodronate, in AAV-RIP-scrambled-treated pregnant mice. The control mice received control liposome instead of clodronate (figure 5C). At G16, we detected a significant reduction in pancreatic macrophages (including islet region) in mice that had received clodronate, compared with those that had received control liposome (figure 5D,E). Clodronate treatment did not cause overt diabetes but induced poorer glucose response (figure 5F), likely resulting from impaired beta-cell growth (figure 5G). The impaired beta-cell growth likely resulted from reduced beta-cell proliferation (figure 5H,I). In order to further clarify the role of PIGF in gestational beta-cell proliferation, we injected recombinant mouse PIGF or control saline solution from the pancreatic duct and examined beta-cell proliferation and intraislet macrophages after 7 days. We detected significant increases in Ki-67+ beta cells and significant increases in islet F4/80+ macrophages (online supplementary figure 4). Furthermore, PIGF did not alter beta-cell proliferation in vitro ($1.5\% \pm 0.2\%$ Ki-67+ beta cells by PIGF vs $1.4\% \pm 0.2\%$ by control saline), reinforcing our conclusion for an importance role of macrophages in PIGF-mediated gestational beta-cell growth.

DISCUSSION

The insulin need increases during pregnancy and is met by increases in insulin production, release and sensitivity.¹ Failure of this compensation may lead to glucose

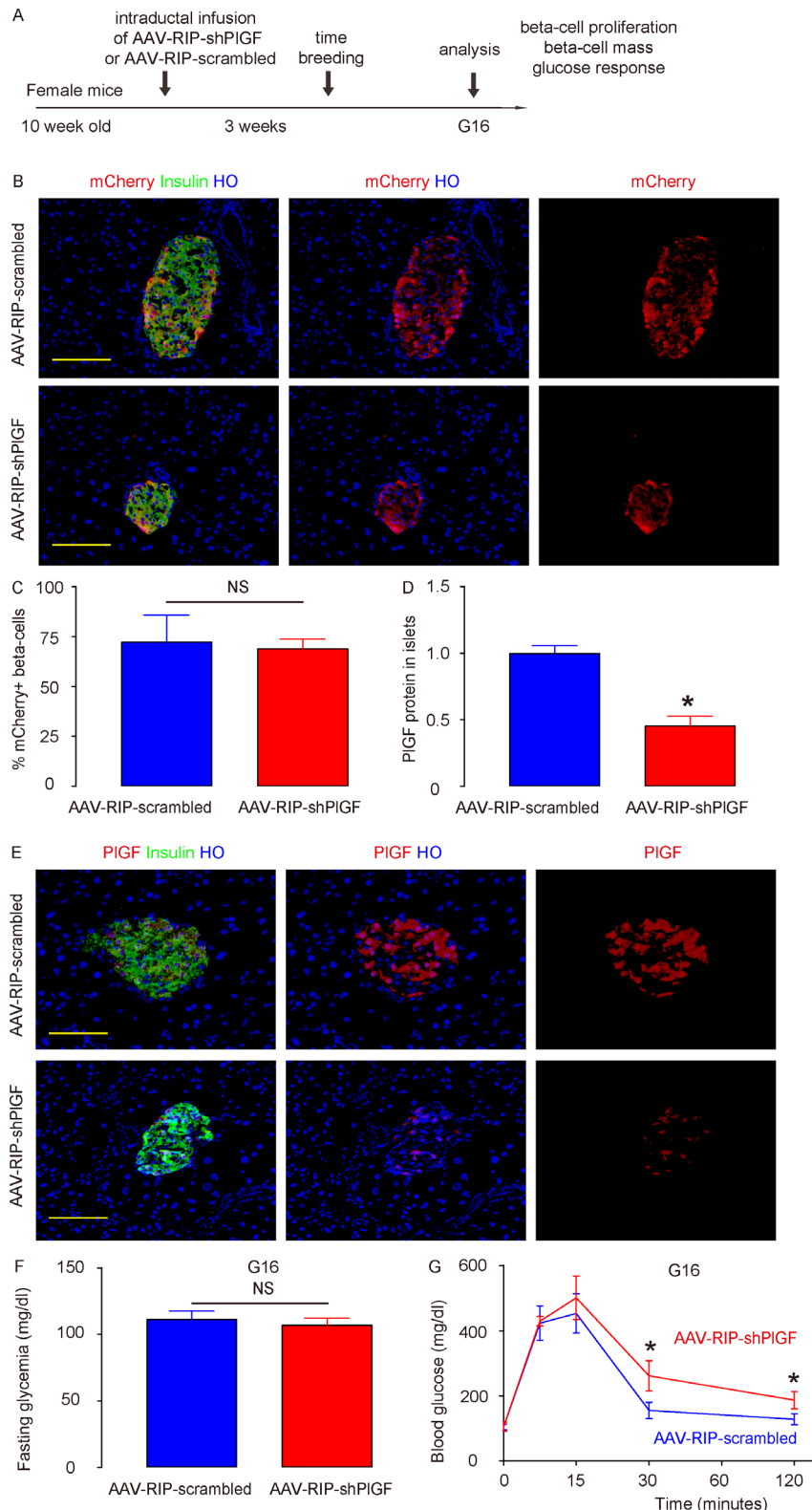


Figure 3 Depletion of PIGF in beta cells impairs the glucose response during pregnancy. (A) Schematic of in vivo study. The AAV-RIP-shPIGF or AAV-RIP-scrambled virus was infused into pancreatic duct to specifically knockdown PIGF in beta cells in female C57BL/6 mice. Three weeks later, these mice were time-mated with male C57BL/6 mice and sacrificed at G16. (B,C) Representative images for mCherry, insulin and HO staining (B), and quantification for % mCherry+ beta cells (C). (D) Quantification for islet PIGF protein levels by ELISA. (E) Representative images for PIGF, insulin and HO staining in G16 pregnant mouse pancreas. (F) Fasting glycemia at G16. (G) IPGTT for pregnant mice at G16. * $p < 0.05$. Five mice were included in each experimental group. Scale bars are 50 μm . AAV, adeno-associated virus; G16, gestational day 16; HO, Hoechst 33342; IPGTT, intraperitoneal glucose tolerance testing; NS, non-significant; PIGF, placental growth factor; RIP, rat insulin promoter; shPIGF, short hairpin small interfering RNA for PIGF.

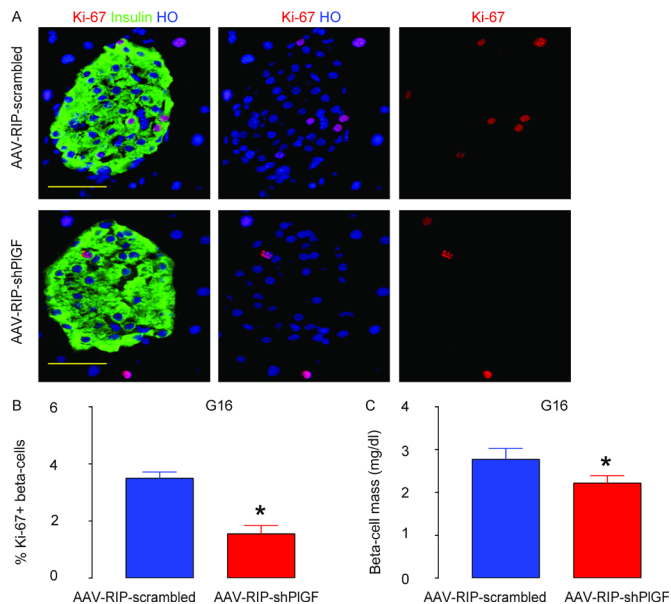


Figure 4 Beta cell-specific knockdown of PIGF inhibits gestational beta-cell proliferation, resulting in attenuated beta-cell growth during pregnancy. (A,B) Ki-67+ beta cells were analyzed in the pancreas from G16 pregnant C57BL/6 mice that had received either AAV-RIP-shPIGF or AAV-RIP-scrambled, shown by representative images (A) and by quantification (B). (C) Beta-cell mass. * $p < 0.05$. Five mice were included in each experimental group. Scale bars are 50 μm . AAV, adeno-associated virus; G16, gestational day 16; HO, Hoechst 33342; RIP, rat insulin promoter; shPIGF, short hairpin small interfering RNA for PIGF.

intolerance or even gestational diabetes, which has been shown to predispose the development of type 2 diabetes.³ Adequate gestational beta-cell growth is critical for maintaining gestational glucose metabolism, which relies on proper production and secretion of pregnancy-associated growth factors.³⁸

Beta cells release many factors directly to pancreas in a paracrine manner, and these factors may act as chemoattractants to recruit the cells that express the corresponding receptors.³⁹ In the current study, PIGF is such a chemoattractant ligand that is produced and secreted exclusively by beta cells in pancreas, while pancreatic macrophages express VEGFR1, the unique receptor for PIGF.²⁰ Although the levels of PIGF in beta cells increased less than twofolds, it is known that the physiological level of a VEGF family member is maintained in a relatively small range,^{17 40} and a modest change may exert significant biological changes.^{41 42} VEGF-A may also play a role in the PIGF-associated effects since VEGF-A regulates vessel permeability, binds VEGFR1 similarly like PIGF, and even forms heterodimers with PIGF.^{43 44} A delicate interaction between VEGF-A and PIGF may coordinate the recruitment of pancreatic macrophages into the islet niche in a tightly organized manner, likely involving multidirectional crosstalk among beta cells, islet endothelial cells and pancreatic macrophages.²⁷ Of note, we have previously shown that VEGFR1 is also expressed in islet endothelial cells.¹⁸ Hence, PIGF/VEGFR1 signaling

may also regulate the restructure of islet endothelia necessary for circulated monocytes/macrophages to enter the islet niche and to join to the tissue-resident macrophages.

The major source of PIGF during pregnancy is the placenta.⁴⁵ It is well known that beta cells locate in a niche where they directly contact with islet endothelial cells.¹⁵ The placenta-derived PIGF in circulation needs to cross the vascular endothelial cells to enter pancreatic parenchyma and attract VEGFR1-expressing macrophages in pancreas. Due to the expression of VEGFR1 on vascular endothelial cells, the majority of placenta-derived PIGF will be 'consumed' in the endothelial cells before entering the pancreatic zone. On the other hand, the beta cell-derived PIGF is likely released into pancreatic parenchyma in a paracrine manner, which attracts pancreatic macrophages that harbor the VEGFR1.

The basal expression of PIGF is much lower than VEGF-A in beta cells. PIGF does not likely play a critical role in baseline postnatal beta-cell homeostasis, since infusion of AAV-RIP-shPIGF did not alter beta-cell mass and glucose response of the mice. However, PIGF did play a critical role in postnatal beta-cell growth in response to increased metabolic need during pregnancy. The increase in PIGF levels in beta cells during pregnancy is only about 50%. However, previous studies have shown that the biological effects of the angiogenic factors are extremely narrow.^{17 40–42} For example, inactivation of only one allele of VEGF-A resulted in embryonic lethality at midgestation,^{46 47} while a twofold increase in VEGF-A levels also led to embryonic lethality.⁴⁸ Therefore, the seemingly modest increase in PIGF levels in beta cells can exert a significant biological effect. The reduction in gestational growth in beta-cell mass by shPIGF was significant but not huge. Nevertheless, it did result in impaired glucose response, suggesting that the typical growth in beta-cell mass during pregnancy may be immediately enough for maintaining proper metabolic requirement without much reserve, which is consistent with the clinical reports showing that some women with gestational diabetes became normoglycemic after pregnancy.²

The intraductal infusion technique allows the pancreas-specific delivery of AAVs, as we have demonstrated in previous reports.^{19 21 24 25 31 49} The AAV-mediated beta cell-specific depletion of PIGF has an advantage over use of beta cell-specific PIGF knockout mice, which are even not available worldwide. Using RIP-Cre to knock out PIGF in beta cells will developmentally alter beta-cell phenotype. On the other hand, using RIP-creERT to knock out PIGF cannot bypass using tamoxifen, which binds to and signals through estrogen receptor highly expressed in beta cells to cause off-target effects.^{50 51} Although here we have shown that PIGF is exclusively produced by beta cells among all pancreatic cells, some inflammatory cells in the pancreas also express certain levels of PIGF.⁵² Thus, our use of the insulin promoter to drive expression of shPIGF assured the absence of effects from the knockdown of PIGF in non-beta cells.

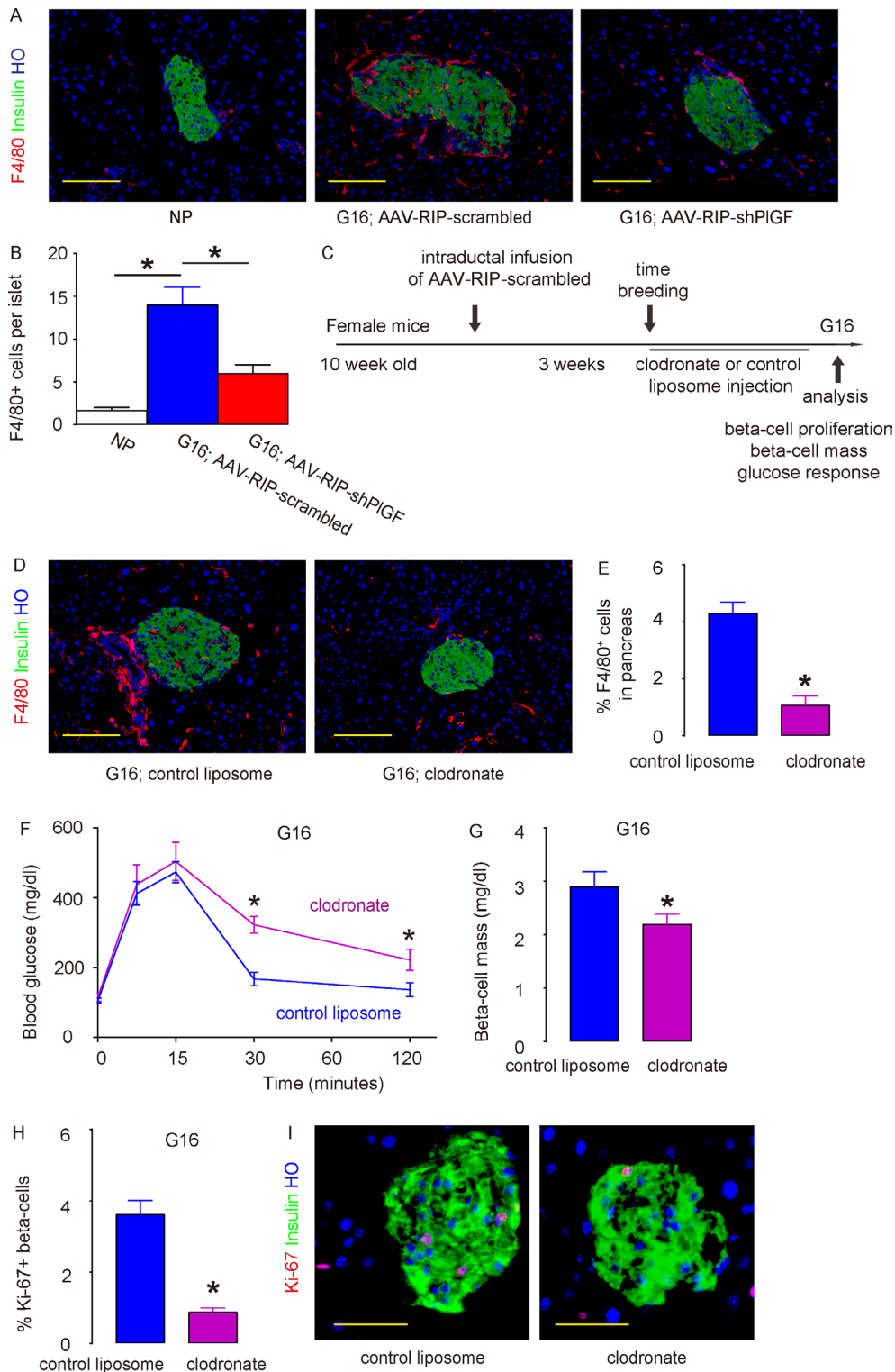


Figure 5 PIGF promotes gestational beta-cell proliferation through recruitment of macrophages. (A,B) Representative images for immunostaining for F4/80 and insulin in NP mouse pancreas, pregnant mouse at G16 treated with AAV-RIP-shPIGF or AAV-RIP-scrambled virus (A) and quantification for intra-islet macrophage number (B). (C) Schematic of macrophage depletion study. The AAV-RIP-scrambled virus was infused into the pancreatic duct in female C57BL/6 mice. Three weeks later, these mice were time-mated with male C57BL/6 mice and received either clodronate or control liposome until sacrifice at G16. (D,E) Representative images for F4/80, insulin and HO staining (D) and quantification of F4/80+ cells in pancreas (E). (F) IPGTT for pregnant mice at G16. (G) Beta-cell mass. (H,I) Ki-67+ beta-cells were analyzed in the pancreas from G16 pregnant C57BL/6 mice that had received either clodronate or control liposome, shown by quantification (H) and by representative images (I). * $p < 0.05$. Five mice were included in each experimental group. Scale bars are 50 μm . AAV, adeno-associated virus; G16, gestational day 16; HO, Hoechst 33342; IPGTT, intraperitoneal glucose tolerance testing; NP, non-pregnant; RIP, rat insulin promoter; shPIGF, short hairpin small interfering RNA for PIGF.

The beta-cell-trophic factors that are produced and secreted by macrophages will be interesting targets to be studied in the future. Previous studies have suggested that WNT ligands,^{32 33} VEGF-A,¹⁶ and combined epidermal growth factor and transforming growth factor $\beta 1$ ²¹ may be such candidates. These studies also highlighted M2 macrophages, an alternatively polarized subtype of macrophages (as opposed to the classical M1 macrophages), as a very important regulator of beta-cell proliferation. It would thus not be surprising if the recruited macrophages to the beta cells during pregnancy are predominantly M2 macrophages.

MIP-GFP (mouse insulin promoter driving a green fluorescent protein reporter) mice have been commonly used for purification of beta cells.⁵³ However, recent studies showed that the presence of human growth hormone (hGH) in the transgenic mice, including MIP-GFP, appeared to affect beta-cell function and proliferation through prolactin receptor signaling.^{54 55} Thus, INS1^{Cre}; Tomato mice, with the absence of hGH in either parental strain, were used to purify beta cells and non-beta islet cells in the current study.

Here, we presented a novel model for studying cell-cell communication in beta-cell proliferation. Our study suggests that increased expression of PIGF in beta cells may trigger gestational beta-cell growth through recruited macrophages. It may be intriguing to examine a possible contribution of insufficient beta cell-derived PIGF to the development of gestational diabetes in future studies.

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