Islet pericytes are required for beta-cell maturity

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Abstract
Beta-cells rely on the islet microenvironment for their functionality and mass. Pericytes, along with endothelial cells, make up the dense islet capillary network. However, while the role of endothelial cells in supporting beta-cell homeostasis is vastly investigated, the role of pericytes remains largely unknown. Here, we focus on pericytes' contribution to beta-cell function. To this end, we employed a transgenic mouse system that allows Diphtheria Toxin-based depletion of pericytes. Our results indicate that islets depleted of their pericytes have reduced insulin content and expression. Additionally, isolated islets displayed an impaired glucose-stimulated insulin secretion, accompanied by a reduced expression of genes associated with beta-cell function. Importantly, reduced levels of the transcription factors MafA and Pdx1 point to beta-cell dedifferentiation in the absence of pericytes. Ex vivo depletion of pericytes in isolated islets resulted in a similar impairment of gene expression, implicating their direct, blood-flow independent role in maintaining beta-cell maturity. To conclude, our findings suggest that pericytes are pivotal components of the islet niche, which are required for beta-cell maturity and functionality. Abnormalities of islet pericytes, as implicated in type 2 diabetes, may therefore contribute to beta-cell dysfunction and disease progression.
**Introduction**

Beta-cell function and maturity are regulated by multiple interactions with cells in their microenvironment (1). In addition to endocrine cells, neuronal, immune, and endothelial cells were shown to support beta-cell gene expression and proliferation (1,2). Islets are highly vascularized by a dense capillary network composed of endothelial cells and pericytes (3,4). However, while the role of endothelial cells in beta-cell homeostasis is vastly investigated, the role of pericytes remains largely unknown (4-6).

Pericyte abnormalities, including their selective loss, play a key role in diabetic retinopathy (7). In islets, pericytes display phenotypic alterations in response to an increased metabolic demand. Hypertrophy of islet pericytes, which accompanies vessel dilation, has been reported in obesity (8,9). Furthermore, abnormalities in pericytes were suggested to contribute to islet fibrosis in rodent models of obesity and type 2 diabetes (10,11). However, the contribution of diabetes-associated changes in islet pericytes to disease progression remains unknown. This stems, in part, from our limited understanding of pericytes' role(s) in glucose homeostasis.

Here, we analyse the role of islet pericytes in beta-cell function. To this end, we utilized an iDTR (inducible Diphtheria Toxin Receptor) (12) and Nkx3.2-Cre (13,14) mouse lines to target and deplete islet pericytes. Shortly after in vivo pericyte depletion, we observed impaired glucose-stimulated insulin secretion (GSIS) in mice and isolated islets. We further observed a reduced expression of genes associated with the mature beta-cell phenotype, including insulin and components of the GSIS machinery. Depleting pericytes in isolated islets resulted in a similar impairment, implicating a direct effect on beta-cells. In conclusion, our results suggest that by supporting the mature beta-cell phenotype, islet pericytes are vital for beta-cell function and glucose homeostasis.
Research Design and Methods

Mice

Mice were maintained according to protocols approved by the Committee on Animal Research at Tel Aviv University. Nkx3.2-Cre (Nkx3.2-2^{m1(cre)Wez})(13) mice were a generous gift from Warren Zimmer (Texas A&M). R26R-EYFP (Gt(Rosa)26Sor^{tm1(EYFP)Cos}) and iDTR (Gt(Rosa)26Sor^{tm1(HBEGF)Awai})(12) mice were obtained from Jackson Laboratories. Mice were i.p. injected with 4 ng/gr Diphtheria Toxin (DT; List), 2 mg/gr Dextrose (Sigma) or 0.001 unit/gr Insulin (Lilly) diluted in PBS.

Immunofluorescence

Pancreatic tissues were fixed in 4% paraformaldehyde, cryopreserved, and cut into 11 µm thick sections. They were then stained using antibodies against Desmin (Dako, M0760), F4/80 (AbD Serotec, MCA497), GFAP (Dako, Z0334), Glucagon (Millipore, AB932), Glut2 (Chemicon, AB1342), Insulin (Dako, A0564), NG2 (Millipore, AB5320), PECAM1 (BD, 553370), Pdx1 (Millipore, 07-696), αSMA (Abcam, Ab5694), Somatostatin (Millipore, MAB354) and YFP/GFP (Abcam, Ab13970), followed by AlexaFluor secondary antibodies (Invitrogen). For TUNEL, In Situ Cell Death detection kit (Roche, 11684809910) was used. Images were acquired using SP8 microscope (Leica). For morphometric analysis, islets were imaged using Keyence BZ-9000 microscope (Biorevo) and analyzed using ImageJ software (NIH).

Islet isolation and treatments

Islets were isolated according to standard protocols (15). For Insulin secretion, isolated islets were incubated in KRB buffer containing indicated glucose levels. For imaging, islets were incubated with Hoechst 33342 (Sigma) and analyzed using SP5 microscope (Leica). For cell depletion, islets were cultured in CMRL (Gibco) medium supplemented with 10% FCS and 1 µg/ml DT (Sigma).
Flow-cytometry

Isolated islets were dispersed to single cells using Trypsin (Sigma), and stained with biotinylated anti-PECAM1 antibody (BD, 553371), followed by APC-conjugated Streptavidin (eBioscience). DAPI (Sigma) was used to exclude dead cells. Analysis was performed using Gallios flow-cytometer (Beckman Coutler) and Kaluza software (Beckman Coutler).

Insulin measurements

Islet and pancreatic insulin was extracted by overnight incubation in 0.18M HCl and 70% Ethanol mixture. Levels were determined using mouse enzyme-linked Immunosorbent assay (ELISA; Mercodia and Alpco).

Quantitative PCR

Expression of Ins1 (GGGTCGAGGTGGGCC, CTGCTGGCCTCGCTTGC), Ins2 (GGCTGCGTAGTGGTGGGTCTA, CCTGCTCGCCCTGCTCTT), and MafA (GCTGGTATCCATGTCCGTGC, TGTTTCAGTCGGATGACCTCC) was analyzed using SYBR green assay (Applied Biosystems) and normalized to Cyclophilin (TGCCGCCAGTGCCATT, TCACAGAATTATTCCAGGATTC). Expression of additional genes was analyzed using Taqman assays (Applied Biosystems) and normalized to Cyclophilin.

Statistical analysis

Paired data was evaluated using a Student’s two-tailed t-test.
Results

*Nkx3.2-Cre drives gene expression to islet pericytes*

Pericytes are found in healthy adult islets (8,9). To study the role of islet pericytes, we established a mouse tool allowing for their manipulation — *Nkx3.2-Cre*, which we previously showed to target mesenchymal cells (but no other cell types) in the embryonic and neonatal pancreas (14). To determine *Nkx3.2-Cre* expression pattern in the adult pancreas, we analysed *Nkx3.2-Cre;R26R-EYFP* mice. Our analysis revealed pancreatic YFP-labeled cells with peri-endothelial location and extended cytoplasmic processes, both of which are pericyte characteristics (16)(Fig. 1A,B). Immunofluorescence analysis further indicated these cells were positive for NG2 (Neural/Glial antigen 2), desmin, PDGFRβ (Platelet-Derived Growth Factor Receptor β) and αSMA (α-Smooth Muscle Actin), portraying their pericytic identity (Fig. 1C-F) (8,9,16).

Islet-associated YFP+ cells were negative for GFAP (Glial Fibrillary Acidic Protein), indicating that they are neither pancreatic stellate nor Schwann cells (Fig. 1G)(9,17). Additionally, both immunofluorescent and flow-cytometric analyses indicated that YFP+ cells do not express PECAM1 (Platelet Endothelial Cell Adhesion Molecule 1)(Fig. 1B,H), implicating that they are not endothelial cells. To conclude, our analysis revealed that *Nkx3.2-Cre* targets pericytes, but not other islet cell types.

**Diphtheria toxin (DT)-dependent ablation of pericytes**

To test whether islet pericytes play a role in endocrine function, we set to deplete this cell population using the DTR system. This system was shown to be highly efficient in ablating islet endocrine cells (as alpha- and PYY-expressing cells) with minimal effect on beta-cell function (18-20). To deplete pericytes, *Nkx3.2-Cre;iDTR* (expressing DTR in a Cre-dependent manner (12,14)), as well as non-transgenic (i.e., Cre-negative) control mice, were
i.p. injected with DT. *Nkx3.2*-Cre has both pancreatic (in pericytes and vascular smooth muscle cells (vSMCs); Fig. 1 and Supplementary Fig. 1) and non-pancreatic expression (in the joints and gastro-intestinal mesenchyme (13)). Therefore, determining primary, rather than secondary, effects requires studying short-term events. To this end, mice were analyzed 36 hours after DT administration. At this time point, we observed no abnormalities in mice activity, weight or insulin sensitivity (Supplementary Fig. 2).

Immunofluorescent analysis revealed that DT treatment of *Nkx3.2*-Cre;iDTR mice resulted in a loss of islet pericytes (Fig. 2A and B and Supplementary Fig. 3), while endothelial area remained unchanged (Fig. 2A and C). Of note, we did not observe macrophages infiltration to islets of treated mice (Supplementary Fig. 3). To conclude, the *Nkx3.2*-Cre;iDTR line allows efficient and rapid depletion of islet pericytes.

**In vivo pericytes’ depletion impairs beta-cell function**

To elucidate the role of islet pericytes, we analyzed for potential changes in beta-cell function in DT-treated *Nkx3.2*-Cre;iDTR mice (“*In vivo* DT”; Fig. 3A). Our analysis indicated elevated fasted blood glucose levels and impaired glucose tolerance in DT-treated transgenic mice (Fig. 3B and C). To test whether pericyte depletion affected GSIS independent of blood flow, we analyzed isolated islets. As shown in Figure 3D, islets isolated from DT-treated transgenic mice secreted significantly less insulin in response to high glucose levels compared to control.

Our immunofluorescent analysis revealed normal islet morphology in DT-treated *Nkx3.2*-Cre;iDTR mice (Fig. 3E). In agreement, we could not detect beta-cell death upon pericyte depletion (Supplementary Fig. 3). However, DT-injected *Nkx3.2*-Cre;iDTR mice displayed reduced pancreatic and islet insulin content (Fig. 3F,G). Analyzing gene expression of isolated islets revealed a significant reduction in transcripts levels of both *Ins1* and *Pcsk1*,...
encoding for insulin and Proprotein Convertase 1/3, respectively (Fig. 3H). Reduction in Ins2 transcripts did not reach statistical significance (Fig. 3H). Our results therefore point to an abrogated insulin production in transgenic islets.

As the decrease in insulin secretion in transgenic islets (2.3 fold; Fig. 3D) was more profound than the decrease in insulin content (1.7 fold; Fig. 3G), we analyzed for potential changes in components of the GSIS machinery. Our analysis revealed that the expression of Glut2, Sur1, and Kir6.2 were significantly reduced in Nkx3.2-Cre;iDTR islets (Fig. 3I and J; 36 and 72 hours after DT treatment, respectively). Therefore, pericyte depletion affected beta-cell ability to respond properly to glucose.

Beta-cell maturity has been shown to depend on expression of transcription factors, including MafA and Pdx1 (21). We observed a reduced expression of both of these factors in islets from DT-treated mice (Fig. 3K and L; 36 and 72 hours after DT treatment, respectively). We further observed expression of Hes1, a transcription factor associated with beta-cell dedifferentiation, in transgenic islets (Fig. 3K)(21). To conclude, our results indicate that pericyte depletion may interfere with beta-cell maturity.

**Pericyte depletion in isolated islets impairs beta-cell gene expression**

Adult beta-cell phenotype was recently shown to be independent of blood circulation (22). Nevertheless, as the Nkx3.2-Cre targets also pancreatic vSMCs (Supplemental Fig. 1), we set to deplete pericytes in isolated islets to test their role independent of potential effects on blood flow (Fig. 4A; “Ex vivo DT”). Furthermore, as Nkx3.2-Cre targets pericytes, but no other islet cell types (Fig. 1), this setting allows us to specifically deplete them without affecting other cell types targeted by the Cre (13) (Supplemental Fig. 1).

The pericyte network is maintained upon islet isolation (Fig. 4B), which allows for their DT-mediated ex vivo depletion in islets isolated from untreated Nkx3.2-Cre;iDTR mice
(Fig. 4C). To test for resultant effects of pericyte depletion on beta-cell phenotype, islets were isolated from untreated Nkx3.2-Cre;iDTR and non-transgenic mice, and cultured in the presence or absence of DT. After 24 hours, a significant reduction in the expression level of beta-cell genes was observed in transgenic islets treated with DT ex vivo, including *Ins1*, *Glut2*, *MafA* and *Pdx1* (Fig. 4D). To conclude, our results indicate that pericytes in the islet niche support beta-cell gene expression and maturity.
Discussion

Beta-cell phenotype has been shown to depend on their microenvironment (1). Here, we focus on the contribution of pericytes to beta-cell function. To this end, we depleted these cells by DT, and analyzed the resultant effect on beta-cells. Soon after pericyte depletion, we observed impaired GSIS and insulin content in isolated islets, accompanied by a reduced expression of genes associated with the mature beta-cell phenotype. Ex vivo depletion of islet pericytes resulted in similar impairment in beta-cell gene expression, implicating an intra-islet role. Therefore, our findings suggest that pericytes are pivotal components of the islet niche, as they are required for beta-cell maturity and functionality.

Pericytes were suggested to regulate islet vascular permeability and blood flow by affecting neighboring endothelial cells (8). Hence, the observed dysfunction of beta-cells upon pericyte depletion may potentially be secondary to abrogated vascular function. However, it was recently shown that adult beta-cell function is independent of blood flow, as it is unaffected by ~50% reduction in islet endothelium (22). Furthermore, the impaired beta-cell gene expression upon ex vivo pericyte depletion in isolated islets (Fig. 4) implicates a direct, intra-islet role of these cells in maintaining beta-cell phenotype, independent of blood flow regulation.

The vascular basement membrane (BM) has been shown to promote beta-cell function (6). While endothelial cells contribute to islets’ BM, an additional source was suggested (23). Heterotypic interactions of pericytes and endothelial cells are required for vascular BM assembly in many tissues (16). We recently showed that the pancreatic mesenchyme (which includes pericytes and vSMCs; Fig.1 and Supplemental Fig. 1), but not endothelial cells, produce α2 laminins that support beta-cell maturity in culture (24). Therefore, pericyte depletion may interfere with beta-cell function by disrupting the islet BM. However, the long half-life of BM components does not support the observed rapid changes in beta-cells (Fig. 3
This implicates the involvement of yet unidentified short-lived factors and/or direct cell-cell interactions in pericyte-mediated beta-cell function.

Recent evidence points to beta-cell dedifferentiation as a key in diabetes progression (25). In this process, triggered by misregulation of signaling pathways and/or metabolic stress, beta-cells lose functionality through repression of canonical beta-cell genes (15,21,25). Our results suggest that pericytes are required to maintain beta-cells’ mature, differentiated state. Pericytes have been shown to undergo phenotypic changes in response to increased metabolic demand — a phenomenon that has been implicated in islet fibrosis during diabetes (8,10,11). This raises the possibility that diabetes-associated changes in islet pericytes may interfere with their ability to properly support beta-cells, contributing to dedifferentiation of the latter. Therefore, delineating the pericyte/beta-cell axis would allow for better understanding of the regulation of beta-cell function in health and its loss in diabetes.
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Prior Presentation. This study was presented at the Keystone Symposium on Islet Biology: From Cell Birth to Death, Keystone, CO, 13–18 March 2016.
References


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Figure legends

Figure 1: Nkx3.2-Cre line drives gene expression in islet pericytes

A-G) Pancreatic tissues from adult Nkx3.2-Cre;R26R-EYFP mice were stained for Insulin (Ins; white), YFP (green) and PECAM1 (A,B), NG2 (C), Desmin (D), PDGFRβ (E), αSMA (F) and GFAP (G) (all in red) and counterstained with DAPI (blue). Inserts show higher magnification of the area framed in white box, of all channels (upper), green channel (middle) and red channel (lower). When more than one box is selected, “*” and “#” mark the different boxes. Note islet-associated YFP⁺ cells are positive for all analyzed markers but GFAP and PECAM1. H) Flow-cytometry analysis of islets isolated from non-transgenic (non tg; left) and Nkx3.2-Cre;R26R-YFP (right) mice. Dot plots represent dispersed islet cells stained and analyzed for the endothelial marker PECAM1 (X axis) and yellow fluorescence (Y axis). Numbers represent the percentage of cells positive for both markers (Upper right quarter). Note YFP⁺ cells are negative for PECAM1.
Figure 2: Diphtheria toxin injection to Nkx3.2-Cre;iDTR mice depletes islets pericytes

*Nkx3.2-Cre;iDTR* and non-transgenic (non tg) control mice were i.p. injected with a single dose of 4 ng per gram body weight Diphtheria Toxin (DT) and analyzed after 36 hours. **A)** Pancreatic tissues of transgenic (left) and non-transgenic (right) were stained for NG2 (green) to label pericytes, PECAM1 (red) to label endothelial cells, and insulin to label beta-cells. White lines demark the outer border of Insulin$^+$ area. Note all capillaries in control islets contained both endothelial cells and pericytes, while some capillaries in transgenic islets contains only endothelial cells. Representative fields. **B, C)** Bar diagrams showing decreased islet pericyte area (B), but not endothelial area (C), in transgenic mice (empty bars) as compared to control (black bars). Pancreatic tissues were stained as described in A, and the relative ratio per each islet was calculated. 50-100 islets per mouse, from sections at least 100 $\mu$m apart, were analyzed. N=3. ***P < 0.005, NS = non-significant, as compared to non-transgenic control. Data represent the mean ± SD.
Figure 3: Impaired beta-cell gene expression and glucose-stimulated insulin secretion upon \textit{in vivo} depletion of islet pericytes.

\textit{Nkx3.2-Cre;idTR} (empty circles and bars) and age- and sex-matched non-transgenic controls (non tg; black circles and bars) were injected with a single dose of 4 ng/gr body weight DT, as illustrated (A; \textit{“In vivo DT”}). B) Male mice were i.p. injected with DT and analyzed 36 hours later for blood glucose levels after an overnight fast. N = 7-9. C) Glucose tolerance test were performed 36 hours after DT injection. After overnight fasting, mice were i.p. injected with dextrose (2 mg/g body weight) and blood glucose levels were measured at indicated time. N= 7-9. D) Bar diagram shows impaired glucose-stimulated insulin secretion by isolated transgenic islets. Islets were isolated from mice 36 hours after DT injection and exposed for an hour to 1.67 or 16.7 mM glucose. Secreted insulin levels were measured by ELISA. N = 4. E) Pancreatic tissues of transgenic (right panel) and non-transgenic (left panel) mice 36 hrs after DT injection were stained for insulin (green), glucagon (red) and somatostatin (blue). F, G) Bar diagrams showing reduced pancreatic (F) and islet (G) insulin content in treated transgenic mice. N=4-5. H, I, K) Abnormal beta-cell gene expression in islets isolated from transgenic mice 36 hours after DT injection as compared to control. RNA was extracted and expression of indicated genes was analyzed by qPCR. Note reduced expression of genes required for beta-cell function and maturity, whereas the levels of \textit{Hes1} is elevated in those islets. N=4. J, L) Pancreatic tissues of transgenic (right panel) and non-transgenic (left panel) mice 72 hrs after DT injection were stained for insulin (green) and Glut2 (red; J) or Pdx1 (red; K). The same imaging parameters were used to analyze transgenic and control tissues. Note lower expression of both proteins in transgenic islet. *P < 0.05, **P < 0.01, ***P < 0.005, NS = non-significant, as compared to non-transgenic control. Data represent the mean ± SD.
Figure 4: Impaired beta-cell gene expression upon ex vivo depletion of islet pericytes.

A) A scheme illustrating isolation of islets from untreated mice and their subsequent culturing in media supplemented with DT (+DT; blue), prior to their analysis. B) Unfixed islets isolated from Nkx3.2-Cre;R26R-YFP mice were imaged for YFP (green; right and left panels) and Hoechst (blue; right panel). Picture represents projections of multiple images taken in different focal planes. C,D) Islets isolated from untreated Nkx3.2-Cre;iDTR (striped and empty bars) and non-transgenic controls (non tg; black bars) were cultured in media supplemented with 1 µg/ml DT (+DT; white and black bars) or unsupplemented media (untreated; striped bars) for 24 hours. RNA was extracted and expression levels of indicated genes were analyzed by qPCR. Bar diagrams showing reduced expression of DTR transgene upon treatment, indicated depletion of DTR-expressing cells (C). N=4. *P < 0.05, as compared to untreated transgenic control. Bar diagrams showing reduced expression of beta-cell genes in DT-treated transgenic islets (D). N=4. Representative of three independent experiments. *P < 0.05, **P < 0.01, as compared to treated non-transgenic islets. Data represent the mean ± SD.
Figure 1: Nkx3.2-Cre line drives gene expression in islet pericytes

69x26mm (300 x 300 DPI)
Figure 2: Diphtheria toxin injection to Nkx3.2-Cre;IDTR mice depletes islets pericytes
Figure 3: Impaired beta-cell gene expression and glucose-stimulated insulin secretion upon in vivo depletion of islet pericytes.
Figure 4: Impaired beta-cell gene expression upon ex vivo depletion of islet pericytes

163x302mm (300 x 300 DPI)
Supplemental Figure 1. Nkx3.2-Cre targets gene expression to vascular smooth muscle cells in the pancreas.

Image shows pancreatic tissues of Nkx3.2-Cre;R26-EYFP mouse, stained for YFP (green) to label cells targeted by the Cre, α Smooth Muscle Actin (αSMA; red) to label mural cells (i.e, pericytes and vascular smooth muscle cells), and PECAM1 (magenta) to label endothelial cells. Tissue was counterstained with DAPI (blue). Note vSMCs, defined by both their location around large pancreatic blood vessels and expression of αSMA, are YFP-positive.
Supplemental Figure 2: Insulin sensitivity and weight are maintained in DT-treated Nkx3.2-Cre;iDTR mice.

Nkx3.2-Cre;iDTR (empty bars and circles) and age-matched non-transgenic controls (empty bars and circles) were i.p. injected with 4 ng/gr body weight Diphtheria Toxin (DT) and analyzed 36 hours after treatment. A) Bar diagram represents mouse body weight 36 hours after treatment as compared to their weight before treatment. N=6. B) Insulin tolerance test. Following an overnight fast, control and transgenic mice were i.p. injected with 1 U/kg body weight insulin and their blood glucose levels were measured at indicated time points. N = 8. NS = non-significant, as compared to non-transgenic control. Data represent the mean ± SD.
Supplemental Figure 3. Apoptosis of pericytes is not accompanied by infiltration of immune cells to islet

Pancreatic tissues of Nkx3.2-Cre;iDTR and age-matched non-transgenic controls i.p. injected with 4 ng/gr body weight Diphtheria Toxin (DT) and analyzed 36 hours after treatment. A) Pancreatic tissues of transgenic (middle and right panels) and non-transgenic (left panel) mice were subjected TUNEL assay (green) to identify dying cells, and were stained for NG2 (red) to identify pericytes and insulin (white) to identify beta-cells. Right panels show higher magnification of NG2^+ TUNEL^+ cells found in transgenic islets (marked with blue arrows in middle panel). Note apoptotic NG2^+ pericytes, but not beta-cells, in transgenic islet. B) Images show pancreatic tissues of transgenic (middle panel) and non-transgenic (non tg; left panel) mice, and splenic tissue of non-transgenic mice (staining control; right panel), stained for the mononuclear phagocyte marker F4/80 (green) and Insulin (red) and counterstained with DAPI (blue). Note F4/80^+ cells in splenic but not pancreatic tissues.