Brief Communication

MOLECULAR METABOLISM

Neonatal pancreatic pericytes support β-cell proliferation

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ABSTRACT

Objective: The maintenance and expansion of β-cell mass rely on their proliferation, which reaches its peak in the neonatal stage. β-cell proliferation was found to rely on cells of the islet microenvironment. We hypothesized that pericytes, which are components of the islet vasculature, support neonatal β-cell proliferation.

Methods: To test our hypothesis, we combined *in vivo* and *in vitro* approaches. Briefly, we used a Diphtheria toxin-based transgenic mouse system to specifically deplete neonatal pancreatic pericytes *in vivo*. We further cultured neonatal pericytes isolated from the neonatal pancreas and combined the use of a β-cell line and primary cultured mouse β-cells.

Results: Our findings indicate that neonatal pancreatic pericytes are required and sufficient for β-cell proliferation. We observed impaired proliferation of neonatal β-cells upon *in vivo* depletion of pancreatic pericytes. Furthermore, exposure to pericyte-conditioned medium stimulated proliferation in cultured β-cells.

Conclusions: This study introduces pancreatic pericytes as regulators of neonatal β-cell proliferation. In addition to advancing current understanding of the physiological β-cell replication process, these findings could facilitate the development of protocols aimed at expending these cells as a potential cure for diabetes.

Keywords Beta-cells; Pericytes; Neonatal pancreas; Islets; Vasculature

1. INTRODUCTION

Establishment of β-cell mass is largely dictated during the embryonic and neonatal stages [1–3]. In the embryo, new β-cells are formed by differentiation of pancreatic endocrine precursors, followed by proliferation of differentiated β-cells [3–5]. After birth, the major route of β-cell generation is their replication [1,6,7]. However, β-cell proliferation rates decline with age in both humans and rodents and are significantly higher during the neonatal period than during adulthood [2,3,8–10]. In humans, proliferation of β-cells begins shortly after birth, continues at its highest rate for about a year, and then rapidly declines in early childhood [2,9]. In rodents, the β-cell proliferation rate peaks during the first week of life, and rapidly declines shortly thereafter [3,8]. A further decline in β-cell proliferation rates is observed as humans and rodents age, when the proliferation index of these cells approaches zero during adulthood [2,3,9,11]. However, adult β-cells maintain an ability for compensatory proliferation in response to increased metabolic demand or injury [12–19].

β-cells respond to cues provided by cells of their microenvironment, in which endothelial, neuronal, and immune cells have been shown to promote adult β-cell proliferation [20–24]. In addition to endothelial cells, the dense capillary network of islets contains pericytes, which form a single discontinuous layer around smaller vessels and are intimately associated with endothelial cells [25]. Interactions between endothelial cells and pericytes are required for assembling the vascular basement membrane (BM) [26,27], which, in the islet, was shown to support β-cell proliferation and function [20,28]. Together with vascular smooth muscle cells (vSMCs), which surround large blood vessels, pericytes constitute a class of mesenchymal cells termed 'mural cells' [27]. The embryonic pancreatic mesenchyme was shown by us and others to support the proliferation of pancreatic progenitors and differentiated β-cells [29–35]. After birth, pericytes constitute a major part of the mesenchymal cell population in the pancreas [25,36]. However, the role of pancreatic pericytes in postnatal β-cell proliferation awaits investigation.

Here, we investigated the ability of neonatal pancreatic pericytes to promote β-cell proliferation both *in vitro* and *in vivo*. Our findings indicate that the conditioned medium of cultured neonatal pericytes stimulates the proliferation of both a β-cell tumor line, βTC-tet [37], and primary cultured adult β-cells. Furthermore, pericyte-conditioned medium induced β-cell expansion in an integrin β1-dependent manner, implicating the involvement of BM components in this process. Lastly, we used iDTR (inducible diphtheria toxin [DT] receptor) [38] and *Nkx3.2*-Cre [33,39] mouse lines to target and deplete pericytes in the neonatal pancreas and analyzed the resulting effect on β-cell proliferation. We show that partial pericyte depletion was sufficient

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to reduce the rate of neonatal β-cell proliferation *in vivo*. To conclude, our results point to a pivotal role of pancreatic pericytes in neonatal β-cell proliferation.

2. MATERIALS AND METHODS

2.1. Mice

Mice were maintained according to protocols approved by the Institutional Animal Care and Use Committee at Tel Aviv University. All mice were maintained on a C57BL/6 background. Nkx3.2-Cre (Nkx3-2$^{tm1(cre)Wez}$) [39] mice were a generous gift from Warren Zimmer (Texas A&M). *R26*-YFP (Gt(ROSA)26Sor$^{tm1(EYFP)Cos}$) [40] and iDTR (Gt(ROSA)26Sor$^{tm1(HBEGF)Awai}$) [38] mice were obtained from Jackson Laboratories. Wild-type mice were purchased from Envigo, Ltd. When indicated, mice were i.p. injected with a single dose of 0.25 ng/g body weight Diphtheria Toxin (DT; List) diluted in PBS.

2.2. Islet isolation

Collagenase P (0.8 mg/ml; Roche) diluted in RPMI (Gibco) was injected through the common bile duct into the pancreas of a euthanized adult mouse. Dissected pancreatic tissue was incubated for 10–15 min at 37 °C, followed by a gradient separation with Histopaque 1119 (Sigma) for 20 min at 4 °C. Islets were collected from the gradient interface, followed by their manual collection.

2.3. Flow-cytometry

For cell sorting, dissected pancreatic tissues were digested with 0.4 mg/ml collagenase P (Roche) and 0.1 ng/ml DNase (Sigma) diluted in HBSS for 30 min at 37 °C with agitation, followed by cell filtration [41]. Cells were suspended in PBS containing 5% FCS and 5 mM EDTA and sorted based on their yellow fluorescence by FACS Aria (BD). For staining of cell surface markers, cells were isolated as described above and stained with biotin-conjugated anti-PDGFRβ (Platelet-derived Growth Factor Receptor β) antibody (Catalog #13-1402, Affymetrix) followed by incubation with Allophycocyanin-labeled Streptavidin (Catalog #17-4317-82, Affymetrix). Cells were analyzed by a Gallios cytometer (Beckman Coulter) using Kaluza software (Beckman Coulter). For analysis of cell proliferation, single-cell suspension was obtained by incubating islets with 0.05% Trypsin and 0.02% EDTA solution (Biological Industries) at 37 °C for 5 min with agitation, or by collecting βTC-tet cells with 0.05% Trypsin and 0.02% EDTA solution (Biological Industries). Cells were fixed in 70% ethanol at −20 °C overnight, suspended in PBS containing 1–2% FBS and 0.09% sodium azide, and then immunostained with Fluorescein-conjugated anti-Ki67 (Catalog #11-5698-82, eBioscience or Catalog #556026, BD) antibody. Islet cells were further stained with guinea pig anti-insulin (Catalog #A0564, Dako) antibody, followed by DyLight 650-conjugated secondary antibody (SA5-10097, Invitrogen). For analyzing proliferation rates, cells were analyzed by a FACS Gallios cytometer (Beckman Coulter) using Kaluza software (Beckman Coulter). For cell counting, cells were analyzed by an Accuri C6 cytometer (BD) using its volumetric counting feature.

2.4. Cell culture

For culturing pericytes, at least 1.5×10^5 sorted cells were cultured in DMEM medium (Gibco) containing 10% FCS (Hyclone), 1% L-Glutamine (Biological Industries) and 1% Penicillin-Streptomycin solution (Biological Industries) ('complete DMEM'). Cells were sub-cultured weekly or when about 90% confluent, using 0.25% Trypsin solution with 0.05% EDTA (Biological Industries). Up to their third passage, cells were plated on collagen-coated plates (Catalog #FAL354236, Corning). Media were collected from cells in their fourth passage, passed through a 22 μm filter to exclude cells, supplemented with proteases inhibitor (Roche), and then stored at −80 °C. Islets and βTC-tet cells were grown in complete DMEM. Growth arrest of βTC-tet cells was induced by supplementing culture medium with 1 μg/ml tetracycline (Sigma) for 10 days before a proliferation assay was performed [37,42]. For heat inactivation, pericyte-conditioned medium and complete DMEM were incubated at 62 °C for 20 min. For blocking of β1 integrin signaling, pericyte-conditioned medium was supplemented with either hamster anti-β1 integrin (CD29) antibody (Catalog #555003, BD) or hamster IgM (Catalog #553958, BD) as a control. Cultured pericytes were imaged using a Nikon Eclipse Ti-E epifluorescence inverted microscope.

2.5. Immunofluorescence and morphometric analyses

Dissected pancreatic tissues were fixed in 4% paraformaldehyde for 4 h. Tissue was transferred to 30% sucrose solution overnight at 4 °C, followed by embedding in Optimal Cutting Temperature compound (OCT, Tissue-Tek) and cryopreservation. 11-μm-thick tissue sections were stained with the following primary antibodies: guinea pig anti-insulin (Catalog #A0564, Dako), rabbit anti- αSMA (α smooth muscle actin; Catalog #Ab5694, Abcam), Ki67 (Catalog #RM-9106, Thermo Scientific), and NG2 (Neural Glial antigen 2; Catalog #AB5320, Millipore), and rat anti-PECAM1 (Platelet endothelial cell adhesion molecule 1; Catalog #553370, BD) antibodies, followed by secondary fluorescent antibodies (AlexaFluor, Invitrogen). For TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assays, the Fluorescein In Situ Cell Death Detection Kit (Roche) was used according to manufacturer's protocol. Stained sections were mounted using Vectashield antifade mounting medium with DAPI (Vector). Images were acquired using an SP8 confocal microscope (Leica) or a Keyence BZ-9000 microscope (Biorevo). For analysis of islet endothelial and pericyte coverage, sections at least 50 μm apart were stained as described. Islets were defined as insulin$^+$ areas. NG2$^+$ or PECAM1$^+$ areas within the islets, as well as insulin$^+$ areas, were measured using ImageJ software (NIH). For cell proliferation analysis, sections at least 50 μm apart were stained as described. Images were analyzed manually blind to genotype; at least 300 insulin$^+$ cells were analyzed for each pup.

2.6. Statistical analysis

Paired data were evaluated using Student's two-tailed *t*-test.

3. RESULTS

3.1. Culturing neonatal pancreatic pericytes

In order to test the ability of neonatal pancreatic pericytes to promote β-cell replication *in vitro*, we set out to isolate and culture them. To this end, we sorted YFP-labeled cells from the pancreas of *Nkx3.2*-Cre;*R26*-YFP pups at postnatal day 5 (p5). During development, *Nkx3.2* (*Bapx1*) is expressed in gut, stomach, and pancreatic mesenchyme, as well as in skeletal somites [43,44]. In the embryonic and adult pancreas, the *Nkx3.2*-Cre mouse line specifically targets mesenchymal cells, which, in the adult, consist of pericytes and vSMCs [27,33,36,41]. To determine if the *Nkx3.2*-Cre mouse line targets pancreatic pericytes at the neonatal age, as in adults, we analyzed fluorescently labeled ('Nkx3.2/YFP$^+$') cells of p5 *Nkx3.2*-Cre;*R26*-YFP pancreatic tissue for PDGFRβ, which is expressed on the surface of pericytes but not on that of vSMCs [27]. As shown in Figure 1A, our flow-cytometry analysis revealed that ~90% of Nkx3.2/YFP$^+$ cells in the p5 pancreas express PDGFRβ, displaying their pericytic identity. PDGFRβ-negative Nkx3.2/YFP$^+$ cells represent vSMCs, which are

