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Short Communication

Pancreatic pericytes originate from the embryonic pancreatic mesenchyme

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ABSTRACT

The embryonic origin of pericytes is heterogeneous, both between and within organs. While pericytes of coelomic organs were proposed to differentiate from the mesothelium, a single-layer squamous epithelium, the embryonic origin of pancreatic pericytes has yet to be reported. Here, we show that adult pancreatic pericytes originate from the embryonic pancreatic mesenchyme. Our analysis indicates that pericytes of the adult mouse pancreas originate from cells expressing the transcription factor Nkx3.2. In the embryonic pancreas, Nkx3.2-expressing cells constitute the multilayered mesenchyme, which surrounds the pancreatic epithelium and supports multiple events in its development. Thus, we traced the fate of the pancreatic mesenchyme. Our analysis reveals that pancreatic mesenchymal cells acquire various pericyte characteristics, including gene expression, typical morphology, and periendothelial location, during embryogenesis. Importantly, we show that the vast majority of pancreatic mesenchymal cells differentiate into pericytes already at embryonic day 13.5 and progressively acquires a more mature pericyte phenotype during later stages of pancreas organogenesis. Thus, our study indicates the embryonic pancreatic mesenchyme as the primary origin to adult pancreatic pericytes. As pericytes of other coelomic organs were suggested to differentiate from the mesothelium, our findings point to a distinct origin of these cells in the pancreas. Thus, our study proposes a complex ontogeny of pericytes of coelomic organs.

1. Introduction

Pericytes are vascular mural cells defined by their periendothelial location. They are components of the organs' capillary network, where they extend primary cytoplasmic processes along the endothelial tube (Armulik et al., 2011; Tang et al., 2013). These cells were shown to regulate various physiological events, ranging from vascular morphogenesis and maintenance of the blood-brain barrier to the modulation of tissue inflammation and metastasis formation (Armulik et al., 2011; Murgai et al., 2017; Winkler et al., 2011). In the pancreas, multiple roles for pericytes were recently described, including the local regulation of blood flow and the direct support of proper β-cell function and proliferation (Almaça et al., 2018; Epshtein et al., 2017a; Houtz et al., 2016; Sakhneny et al., 2018b; Sasson et al., 2016). An abnormal phenotype of pancreatic pericytes was further associated with diminished β-cell function during diabetes (Almaça et al., 2018; Richards et al., 2010; Sakhneny et al., 2018b; Tang et al., 2013). Despite their central role in pancreas physiology, the embryonic origin of pancreatic pericytes is unknown.

The embryonic origin of pericytes was shown to be heterogeneous, both between and within organs (Armulik et al., 2011; Dias Moura Prazeres et al., 2017). While pericytes in the heart, lung, liver, and gut originate from the mesothelium, pericytes in the central nervous system and thymus develop from the neural crest (Armulik et al., 2011; Dias Moura Prazeres et al., 2017). In addition to this inter-organ heterogeneity, skin pericytes were recently shown to originate from multiple sources (Yamazaki et al., 2017). Studies on the origin of vascular mural cells in coelomic organs have led to the proposal of a common principle according to which vascular Smooth Muscle Cells (vSMCs) and pericytes of these organs originate from the mesothelium (Armulik et al., 2011; Asahina et al., 2011; Wilm et al., 2005). However, for most tissues, including the pancreas, pericytes ontogeny remained to be uncovered.

The pancreatic mesenchyme comprises cells of splanchnic mesoderm origin that form a multicellular layer surrounding the pancreatic epithelium from the onset of organogenesis (Gittes, 2009). The pancreatic mesenchyme was shown to play a vital role in pancreas organogenesis, by supporting survival, proliferation, migration, and

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branching morphogenesis of the pancreatic epithelium (Sakhneny et al., 2018a). While pancreatic mesenchymal cells were considered to be excluded from the developing pancreas as organogenesis progresses, recent evidence indicates they are present also at the end of gestation, albeit encompassing a smaller portion of the pancreatic tissues (Gittes, 2009; Landsman et al., 2011). Whereas mesenchymal cells of the closely related tissues gut and the stomach become smooth muscle cells of the adult tissue (Wells and Melton, 1999), the fate of the pancreatic mesenchyme is currently unknown.

Here, we show that adult pancreatic pericytes originate from the embryonic pancreatic mesenchyme. Our analysis indicated that the origin of adult pancreatic pericytes are cells expressing the homeobox-containing transcription factor Nkx3.2 (Nkx3-2; also known as BapX1). In the embryonic pancreas, *Nkx3.2* expression is restricted to mesenchymal cells at early stages of organogenesis (Hecksher-Sørensen et al., 2004; Landsman et al., 2011; Lettice et al., 2001). We thus traced the fate of Nkx3.2-expressing pancreatic mesenchymal cells in mouse embryos. Profiling the transcriptome of these cells revealed the progressive expression of pericytes markers during pancreas organogenesis. Interestingly, the vast majority of pancreatic mesenchymal cells co-express the pericytic marker PDGFRβ (Platelet Derived Growth Factor Receptor β) as early as at embryonic day (e) 13.5, indicating these cells are predominantly fated to become pericytes. We further showed that the pancreatic mesenchyme acquires additional pericytic characteristics during pancreas organogenesis, as indicated by their typical morphology and periendothelial location. Thus, our study indicates an early commitment of mesenchymal cells to pericytic fate during pancreas development and point to these cells as the primary origin of pericytes of the adult pancreas.

2. Materials and methods

2.1. Mice

All experiments were performed according to protocols approved by the Committee on Animal Research at Tel Aviv University. *Nkx3.2*-Cre (*Nkx3-2*$^{tm1(cre)Wez}$) (Verzi et al., 2009) were a generous gift from Warren Zimmer (Texas A & M University, TX). *LSL*-YFP (*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$) mice were obtained from The Jackson Laboratory. Noon on the day a vaginal plug was detected was considered as embryonic day 0.5.

2.2. Immunofluorescence

Dissected pancreatic tissues were fixed with Paraformaldehyde (4%) for 4 h, embedded in O.C.T. Compound, and cryo-sectioned to 11 μm sections. Dissected e11.5 embryos were fixed with Z-fix (Anatech) for 16 h, embedded in paraffin wax, and sectioned to 5 μm sections. Sections were stained using primary antibodies (listed in Table S1), followed by staining with AlexaFluor tagged secondary antibodies (Invitrogen). Images were acquired using a Keyence BZ-9000 microscope (Biorevo) and SP8 confocal microscope (Leica).

2.3. Morphometric quantifications

Tissue sections, at least 100 μm apart, were stained with as indicated. Images were acquired using a Keyence BZ-9000 microscope (Biorevo) and analyzed manually; at least 500 NG2$^+$ cells were analyzed for each mouse.

2.4. Flow cytometry

Cells were isolated as described (Epshtein et al., 2017b). For cell sorting, cells were collected using FACS Aria (BD) based on their YFP expression. For cell analysis, cells were stained with biotin-conjugated primary antibody followed by staining with fluorescently-labeled Streptavidin (Table S1) and analyzed using CytoFLEX flow-cytometer (Beckman Coulter) and Kaluza software (Beckman Coulter).

2.5. Quantitative PCR analysis

RNA was extracted from isolated tissue and cells using PureLink RNA micro kit (Invitrogen), according to manufacturer's protocol followed by cDNA generation using high capacity cDNA reverse transcription kit (Invitrogen). Gene expression levels were determined using TaqMan gene specific commercial assays (Invitrogen).

2.6. RNA sequencing

Total RNA was extracted from Nkx3.2/YFP$^+$ cells, sorted by flow cytometry. For each analyzed age, cells isolated from multiple embryos were combined to obtain two independent samples of 200 ng of total RNA each that were used to generate libraries with the Illumina TruSeq RNA sample preparation kit. Each library was diluted to approximately 10 pM before loading and sequenced using a Hi-seq. 2000 instrument generating 50 base pair reads. Library preparation and sequencing was performed by the UC Irvine Genomics High-Throughput Facility Core. The 50 base pair reads were trimmed by five bases at the 5′ end due to quality scores. The trimmed 45 base pair reads were then mapped to the mouse genome (NCBI37/mm9) and exon-exon junctions using TopHat (Trapnell et al., 2009) version 1.3.1 with default parameters. Genome information was downloaded from the UCSC Genome Browser (Dreszer et al., 2012). We filtered TopHat alignments and kept reads (and their locations) that could be mapped uniquely. Per gene, we extracted read counts by using samtools (Li et al., 2009) version 0.1.13. Those were normalized by the length of the gene and the total number of mapped reads in order to get a standardized count (RPKM, reads per kilobase of exon model per million mapped reads).

2.7. Statistical analysis

For the dataset obtained from RNA sequencing, we used the R package edgeR (Robinson et al., 2010) to find differentially expressed genes. EdgeR allows differential expression analysis of RNAseq expression data by implementing a range of statistical methodologies based on negative binomial distributions, including empirical Bayes estimation, exact tests, generalized linear models and quasi-likelihood tests (Robinson et al., 2010). P-values obtained were corrected for multiple testing. A corrected p-value of 0.01 was considered significant. For data obtained from qPCR and flow cytometry analyses, paired data were evaluated using Student's two-tailed *t*-test.

2.8. Data availability

Gene expression data have been deposited in ArrayExpress (accession E-MTAB-7448), and files can be accessed at https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-7448/

3. Results

3.1. Pancreatic pericytes derived from Nkx3.2 expressing cells

Our previous studies showed that both the embryonic pancreatic mesenchyme and adult pancreatic pericytes are targeted in *Nkx3.2*-Cre mice (Epshtein et al., 2017a; Landsman et al., 2011; Sakhneny et al., 2018b; Sasson et al., 2016). While not expressed in e8.5 embryos, *Nkx3.2*-Cre is active in the mesenchymal layer adjacent to pancreatic epithelium at e9.5 (Landsman et al., 2011; Verzi et al., 2009). Thus, the pancreatic mesenchyme, but not its epithelium, expresses YFP in *Nkx3.2*-Cre;*LSL*-YFP embryos (Landsman et al., 2011) (Fig. 1A). In the adult *Nkx3.2*-Cre;*LSL*-YFP mice, pancreatic pericytes (defined by periendothelial location, cell morphology, and markers expression)

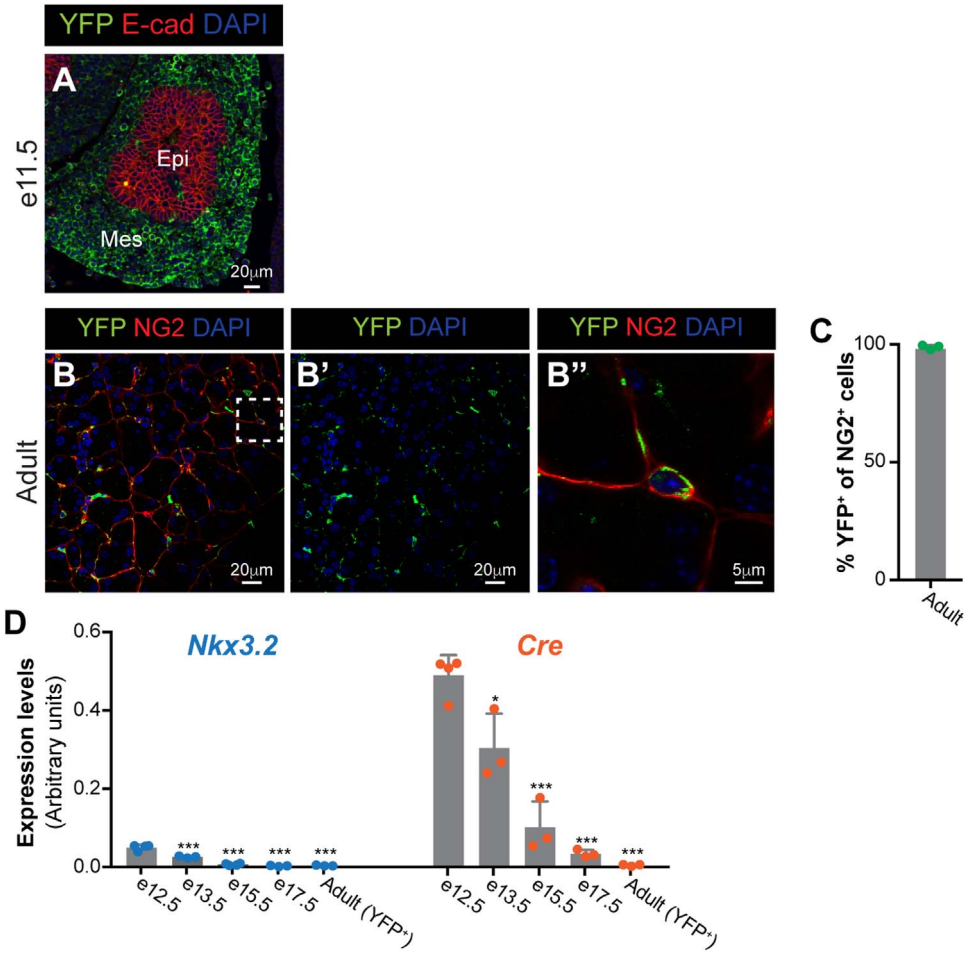

Fig. 1. Adult pancreatic pericytes derived from *Nkx3.2* expressing cells. *Nkx3.2*-Cre;*LSL*-YFP embryos and mice were analyzed. **A**) Immunofluorescence analysis of pancreatic tissue from e11.5 embryos for YFP (green), the epithelial marker E-cadherin (red), and DAPI (blue). Mes, mesenchyme; Epi, epithelium. **B**) Immunofluorescence analysis of pancreatic tissue from adult mice for YFP (green), the pericytic marker NG2 (red), and DAPI (blue). B′, shows only the green and blue channels of the image shown in B. B′′, Shows higher magnification of the area framed in a white box in B. **C**) Morphometric analysis of dissected adult pancreatic tissues stained as described in B. Bar diagram shows the mean percentage (± SD) of YFP$^+$ cells out of analyzed pancreatic NG2$^+$ cells, dots represent the obtained percentage in each mouse. N = 3. **D**) qPCR analysis of *Nkx3.2* gene and *Cre* transgene expression. RNA was extracted from whole dissected embryonic tissues and sorted YFP$^+$ cells from adult pancreata. Gene expression levels were normalized to *Des*, encodes desmin, to normalize for the different portion of pancreatic mesenchymal cells at each of the analyzed ages (Landsman et al., 2011). Bar diagram shows mean ± SD, dots represent the obtained expression levels in each sample. N = 3–4. *, p < 0.05, ***, p < 0.005 as compared to e12.5 (Student's *t*-test).

express YFP (Sakhneny et al., 2018b; Sasson et al., 2016)(Fig. 1B). To determine the portion of pericytes that are targeted by the *Nkx3.2*-Cre transgene, we quantified the fraction of these cells (identifying by the expression of the pericytic marker NG2 [Neuron-glial antigen 2]) that co-express YFP in adult *Nkx3.2*-Cre;*LSL*-YFP pancreata. As shown in Fig. 1C, our morphometric analysis revealed that close to all (98.9% ± 0.95) of pancreatic pericytes are labeled with YFP.

In the *Nkx3.2*-Cre mouse line, a cassette encoding Cre transgene was inserted into exon 1 of the endogenous gene, allowing the targeting of Nkx3.2-expressing cells and their progenies (Verzi et al., 2009). As Cre-mediated recombination is an irreversible process, cells expressing YFP in *Nkx3.2*-Cre;*LSL*-YFP transgenic mice (from herein referred to as Nkx3.2/YFP$^+$ cells) may either actively express Nkx3.2, and hence Cre, or are derived from progenitors that express it. To differentiate between these two options, we analyzed *Nkx3.2* and Cre mRNA expression in pancreatic tissues of *Nkx3.2*-Cre;*LSL*-YFP embryos at various ages (ranging from e12.5 to e17.5) and purified Nkx3.2/YFP$^+$ cells from pancreatic tissues of adult *Nkx3.2*-Cre;*LSL*-YFP transgenic mice. In an agreement with previous studies, *Nkx3.2* expression was detected at early stages on pancreas organogenesis (e12.5 and e13.5; Fig. 1D), and declines thereafter (Hecksher-Sørensen et al., 2004; Lettice et al., 2001) (Fig. 1D). *Nkx3.2* expression levels were below detection in e17.5 pancreatic tissues and adult Nkx3.2/YFP$^+$ pancreatic cells (Fig. 1D). Thus, while *Nkx3.2* is expressed in early embryonic pancreatic tissues, its expression declines at later embryonic ages and is absent from the adult tissue. In correspondence with the *Nkx3.2* expression pattern, the pancreatic expression levels of Cre mRNA decline with age, and were below detection in adult Nkx3.2/YFP$^+$ pancreatic cells (Fig. 1D). Of note, while its expression is controlled by the endogenous *Nkx3.2* promotor in *Nkx3.2*-Cre mice (Verzi et al., 2009), the expression levels of the *Cre* transgene were higher than the levels of *Nkx3.2* gene (Fig. 1D), likely representing the higher stability of the transgenic mRNA.

Summarily, our analysis shows the expression of the *Nkx3.2* gene is restricted to the embryonic pancreas. Thus, while pancreatic pericytes are YFP-labeled in adult *Nkx3.2*-Cre;*LSL*-YFP mice they express neither the *Nkx3.2* gene nor the Cre transgene, indicating they instead derived from cells expressing these genes.

3.2. Nkx3.2/YFP$^+$ cells represent cells of the embryonic pancreatic mesenchyme

In the embryonic pancreas, *the Nkx3.2* expression is restricted to mesenchymal cells (Hecksher-Sørensen et al., 2004; Landsman et al., 2011; Lettice et al., 2001). Because pancreatic pericytes originate from *Nkx3.2*-expressing cells, we hypothesized that the embryonic pancreas

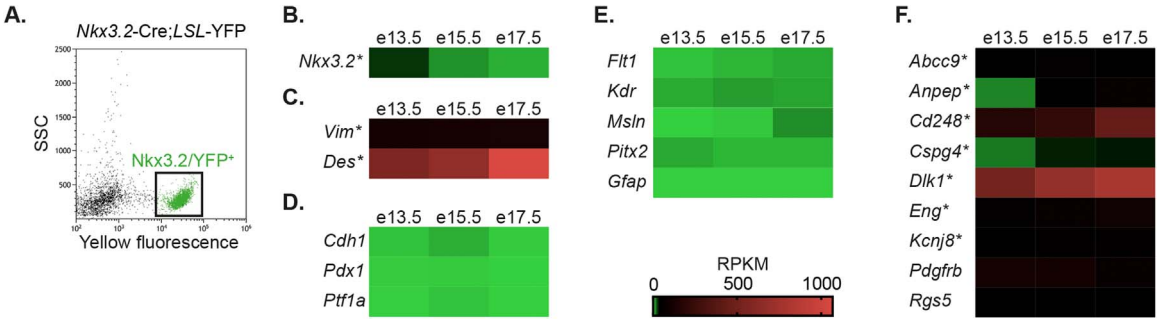

Fig. 2. Embryonic Nkx3.2/YFP$^+$ pancreatic cells express mesenchymal and pericytic genes. Dissected pancreatic tissues from e13.5, e15.5, and e17.5 *Nkx3.2*-Cre;*LSL*-YFP embryos were digested and sorted by flow cytometry to obtain purified Nkx3.2/YFP$^+$ cells, followed RNAseq analysis of sorted cells. **A**) A dot plot is showing the presence of YFP-positive cell population (gated cells; 'Nkx3.2/YFP$^+$ cells') in the embryonic pancreas. **B**–**F**) Heat maps show mean expression levels (as RPKM) of indicated genes at each of the three analyzed ages. Shown are expression levels of *Nkx3.2* (B); pan-mesenchymal (C); pancreatic epithelial (D); endothelial, mesothelial, and PaSC (E); and pericyte (F) genes. N = 2. * marks genes showing reliable expression (> 20 RPKM) and statistically significant differences (p-value < 0.01; calculated using the statistical software edgeR (Robinson et al., 2010)) between the 17.5 and 13.5.

mesenchymal cells differentiate to pericytes. To test this hypothesis, we set out to profile mesenchymal gene expression at various stages of pancreas development, from mid to late stage of organogenesis. We profiled the gene expression of Nkx3.2/YFP$^+$ cells isolated from pancreatic tissues of e13.5, e15.5, and e17.5 *Nkx3.2*-Cre;*LSL*-YFP embryos. Nkx3.2/YFP$^+$ cells were sorted by flow cytometry from embryonic pancreata based on their fluorescent labeling and subjected to RNA sequencing (Fig. 2A; https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-7448/). As expected from our qPCR analysis, the level of *Nkx3.2* transcripts significantly dropped after e13.5 (Figs. 1D and 2B). Our transcriptome analysis showed the embryonic Nkx3.2/YFP$^+$ cells expressed the mesenchymal genes *Vim* and *Des* (encoding Vimentin and Desmin, respectively) but did not express the pancreatic epithelial genes *Cdh1*, *Pdx1*, and *Ptf1a* (encoding E-Cadherin, Pdx1, and Ptf1a, respectively; Fig. 2C,D). This analysis confirmed that, as predicted by the known *Nkx3.2* expression pattern (Hecksher-Sørensen et al., 2004; Landsman et al., 2011; Lettice et al., 2001), Nkx3.2/YFP$^+$ cells represent cells of the pancreatic mesenchyme but not of the pancreatic epithelium.

3.3. The pancreatic mesenchyme gradually expresses pericyte genes during embryogenesis

Next, we analyzed the expression profile of Nkx3.2/YFP$^+$ mesenchymal cells to determine their cell fate acquisition. Our analysis indicates these cells do not express *Flt1*, *Kdr*, *Msln*, *Pitx2*, and *Gfap* genes (encoding VEGFR1 [Vascular Endothelial Growth Factor Receptor 1], VEGFR2, Mesothelin, Pitx2 [Paired-like homeodomain transcription factor 2], and GFAP [Glial Fibrillary Acidic Protein], respectively; Fig. 2E), indicating they do not acquire endothelial, mesothelial or pancreatic stellate (PaSC) cell fate during embryogenesis. These results agree with our previous findings, showing that the *Nkx3.2*-Cre transgene targets neither pancreatic endothelial cells nor PaSCs (Landsman et al., 2011; Sasson et al., 2016).

To test our hypothesis that embryonic Nkx3.2/YFP$^+$ mesenchymal cells give rise to pancreatic pericytes, we set to determine if these cells express pericytic genes. We focused on nine genes previously shown to expressed by pericytes (Armulik et al., 2011): *Abcc9*, *Anpep, Cd248, Cspg4*, *Dlk1*, *Eng*, *Kcnj8*, *Pdgfrb*, and *Rgs5* (encoding: Sur2, Anpep [alanyl (membrane) aminopeptidase], Endosialin, NG2, Dlk1 [Delta Like Non-Canonical Notch Ligand 1], Endoglin, Kir6.1, PDGFRβ, and RGS5 [Regulator of G-Protein Signaling 5]). Among these genes, all but *Anpep* and *Cspg4* were expressed by Nkx3.2/YFP$^+$ cells at considerable levels (RPKM > 20) already at e13.5, pointing to the acquisition of pericytic fate relatively early during pancreas development (Fig. 2F). All nine genes were expressed by these cells at e15.5 and e17.5 when the levels of all but *Pdgfrb* and *Rgs5* were significantly higher at e17.5 than at e13.5 (Fig. 2F). Thus, our analysis revealed that Nkx3.2/YFP$^+$ pancreatic mesenchymal cells acquire pericytic fate, but not alternative mesenchymal fates, during pancreas development. Furthermore, our findings point to a gradual acquisition of pericytic phenotype by mesenchymal cells during pancreas organogenesis.

3.4. The bulk of pancreatic mesenchymal cells express the pericytic marker PDGFRβ at e13.5

Next, we aimed to determine the portion of mesenchymal cells that acquire a pericytic phenotype. To this end, we analyzed the expression of PDGFRβ, required for pericyte recruitment to blood vessels (Armulik et al., 2011; Song et al., 2005), by pancreatic mesenchymal cells of the Nkx3.2/YFP lineage. While our gene expression analysis indicates that the pancreatic mesenchyme expresses the encoded gene, *Pdgfrb*, at e13.5 (Fig. 2F), previous gene expression analysis points to its expression by these cells as early as at e11.5 (Guo et al., 2013).

To analyze for PDGFRβ protein expression by mesenchymal cells, pancreatic tissues of *Nkx3.2*-Cre;*LSL*-YFP embryos were stained for this receptor. As shown in Fig. 3A, our immunofluorescence analysis revealed that PDGFRβ is expressed throughout the Nkx3.2/YFP$^+$ cell population at e13.5, when expressing cells formed a multilayer surrounding the pancreatic epithelium. To determine the percentage of Nkx3.2/YFP$^+$ cells expressing this receptor we performed a flow cytometry analysis, allowing a quantitate measurement of co-expressing cells, on pancreatic tissues from e12.5 and e13.5 embryos (Fig. 3B). While at e12.5 about two thirds (67.7% ± 5.5) of Nkx3.2/YFP$^+$ mesenchymal cells express PDGFRβ, 98.5% ± 0.8 of these cells expressed this pericytic marker at e13.5 (Fig. 3B). This analysis suggests that nearly all Nkx3.2/YFP$^+$ mesenchymal cells acquire pericyte fate at e13.5. The spatial distribution and abundance of pancreatic PDGFRβ$^+$ cells further indicate these cells represent the primary pancreatic mesenchymal cell population, rather than its mesothelium (Fig. 3). To conclude, our analysis points to pericytes as the predominant fate of the embryonic pancreatic mesenchyme.

3.5. Embryonic pancreatic mesenchymal cells display pericytic morphology and periendothelial location

In addition to marker expression, pericytes are defined by the extension of primary cytoplasmic processes and their periendothelial location (Armulik et al., 2011). We, therefore, set to determine the morphology and location of pancreatic Nkx3.2/YFP$^+$ cells at various stages of pancreas organogenesis. Cytoplasmic processes can be detected in Nkx3.2/YFP$^+$ cells beginning of e13.5, which at this age were more evident in cells adjacent to epithelial cells than in cells located at the pancreas periphery (Fig. 4A). At e15.5 and e17.5,

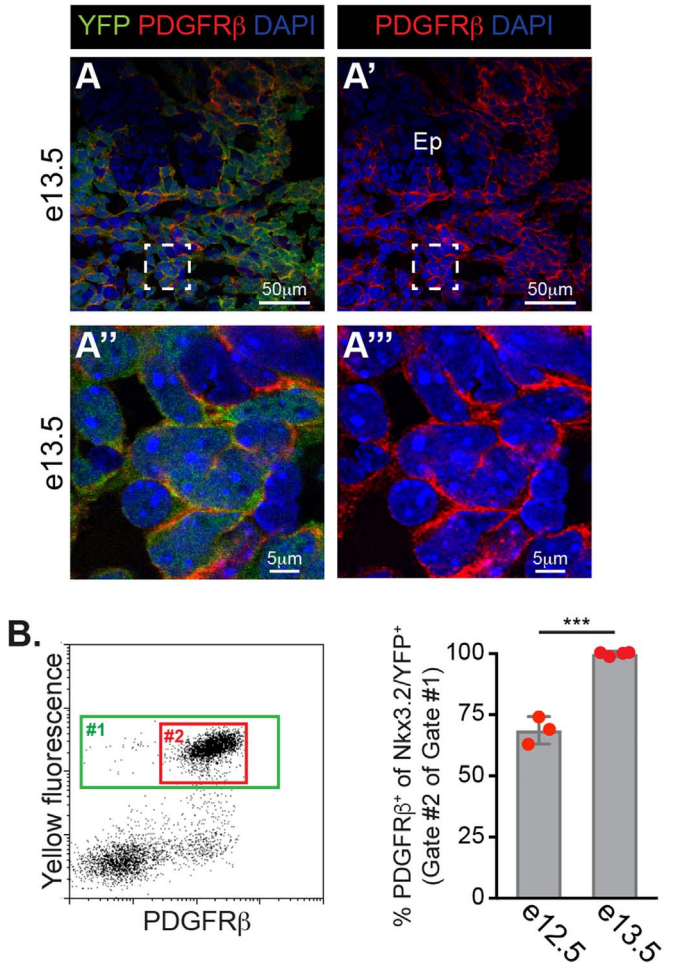

Fig. 3. The vast majority of pancreatic mesenchymal cells express PDGFRβ at e13.5. Dissected pancreatic tissues from *Nkx3.2*-Cre;*LSL*-YFP embryos were analyzed. **A**) Immunofluorescence analysis of e13.5 pancreatic tissue for YFP (green), PDGFRβ (red), and DAPI (blue). A′ shows only the red and blue channels of the image shown in A. A′′ and A′′′ show higher magnification of the areas framed in white boxes in A and A′, respectively. Epi, epithelium. **B**) Flow cytometry analysis of e12.5 and e13.5 pancreatic tissues. Left, A representative dot plot shows PDGFRβ (x-axis) and yellow fluorescence (y-axis) expression. All Nkx3.2/YFP$^+$ cells are gated in gate #1 (green box), and Nkx3.2/YFP$^+$ PDGFRβ$^+$ are gated in gate #2 (red box). Right, the percentage of Nkx3.2/YFP$^+$ PDGFRβ$^+$ cells (gate #2) out of Nkx3.2/YFP$^+$ cell population (gate #1) was determined. Bar diagram shows mean ± SD, dots represent the obtained percentage in each analyzed embryo. N = 3–4. ***, $p < 0.005$ (Student's *t*-test).

Nkx3.2/YFP$^+$ cells displayed an extended, fibrocyte-like morphology (Fig. 4B, C). Thus, our analysis indicates that Nkx3.2/YFP$^+$ mesenchymal cells in the embryonic pancreas displayed a pericytic morphology.

In the adult pancreas, Nkx3.2/YFP$^+$ pericytes align endothelial cells (Sasson et al., 2016). To determine if Nkx3.2/YFP$^+$ mesenchymal cells in the embryonic pancreas also have a periendothelial location, we co-stained pancreatic tissues of *Nkx3.2*-Cre;*LSL*-YFP embryos for YFP and the endothelial cell marker PECAM1. Our analysis revealed Nkx3.2/YFP$^+$ mesenchymal cells adjacent to endothelial cells at e13.5 (Fig. 4D–F). Similarly to the adult (Sasson et al., 2016), Nkx3.2/YFP$^+$ cells of e17.5 and e15.5 embryos were predominantly localized in proximity to endothelial cells (Fig. 4D–F). This observation indicates that Nkx3.2/YFP$^+$ mesenchymal cells displayed the main pericytic characteristic: periendothelial location. The differences observed in pericytes' periendothelial location between e13.5 and e17.5 pancreata further points to a gradual acquisition of pericytic fate by pancreatic mesenchymal cells. Thus, Nkx3.2/YFP$^+$ mesenchymal cells acquire pericyte characteristics, including their typical morphology and periendothelial location, during embryonic development.

4. Discussion

Our analyses indicate that the pancreatic mesenchyme acquires pericyte fate during embryogenesis, as indicated by their gene and protein expression, morphology, and location. Our findings point to the main pancreatic mesenchymal cell population as the origin of pericytes and indicate pericytes as the primary fate of this population. While pancreatic mesenchymal cells display pericyte characteristics midway through pancreas organogenesis, our analysis suggests a gradual acquisition of a more mature pericyte fate during development.

It has been proposed that coelomic organs' mesothelium layer is the origin of the tissue mesenchymal cells, including fibroblasts, vSMCs, and pericytes (Armulik et al., 2011; Asahina et al., 2011; Wilm et al., 2005). Thus, mesothelial cells that line the coelomic cavities and its organs delaminate and migrate to produce their mesenchymal components (Rinkevich et al., 2012). Importantly, our findings suggest that in the pancreas, pericytes originate from the multi-layered pancreatic mesenchyme rather than from its mesothelium. In contrast to the pancreatic mesenchyme, the pancreas mesothelium is a membrane-forming, non-adhesive single-layer squamous epithelium, which is loosely associated with the surrounded organ (Byrnes et al., 2018; Lewis, 1923). In the developing pancreas, cells of the mesothelium lineage were shown to represent a relatively small cell population that migrate to the pancreatic bud around e8 and is defined by the expression of Mesothelin and Pitx2 (Angelo and Tremblay, 2018; Byrnes et al., 2018). Thus, the embryonic Nkx3.2/YFP-labeled pancreatic mesenchyme is distinct from the mesothelium in its abundance, cellular morphology and organization, gene expression, and location. We, therefore, propose that pancreatic pericytes do not follow the common principle proposed based on other coelomic organs, thus suggesting a more heterogenous ontogeny of vascular mural cells.

In a recent study analyzing the single cell transcriptome of the embryonic pancreas, a pseudo-time estimation proposed the mesothelium as an origin of pancreatic vSMCs (Byrnes et al., 2018). In contrast, vSMCs in the pancreas were shown to differentiate from pericytes (Armulik et al., 2011; Song et al., 2005), and we previously reported that vSMCs are targeted by the *Nkx3.2*-Cre mouse line (Sasson et al., 2016). Thus, it is plausible that similarly to vSMCs of other organs (Armulik et al., 2011), pancreatic vSMCs arise from multiple cellular origins, including the mesothelium and pancreatic mesenchyme-derived pericytes.

Our findings indicate that the differentiation of the pancreatic mesenchyme to pericytes occurs around at the same embryonic age when epithelial cells become committed to the endocrine or exocrine lineages (around e13.5) (Cheng Pan and Wright, 2011; Romer and Sussel, 2015). The acquisition of a more mature pericyte phenotype occurs in parallel to epithelial cell differentiation and maturation (around e15.5 and e17.5) (Cheng Pan and Wright, 2011; Romer and Sussel, 2015). Thus, our study raises the possibility of coordinated development of the pancreatic mesenchyme and epithelium. It is therefore plausible that epithelial-mesenchymal interactions play different roles during various stages of pancreas development, to accommodate the different needs of precursors and more differentiated cells. A better understanding of the molecular basis of this dynamic process is essential in elucidating the events that drive pancreas organogenesis.

5. Conclusions

The embryonic pancreatic mesenchyme is the primary source of pericytes of the adult organ.

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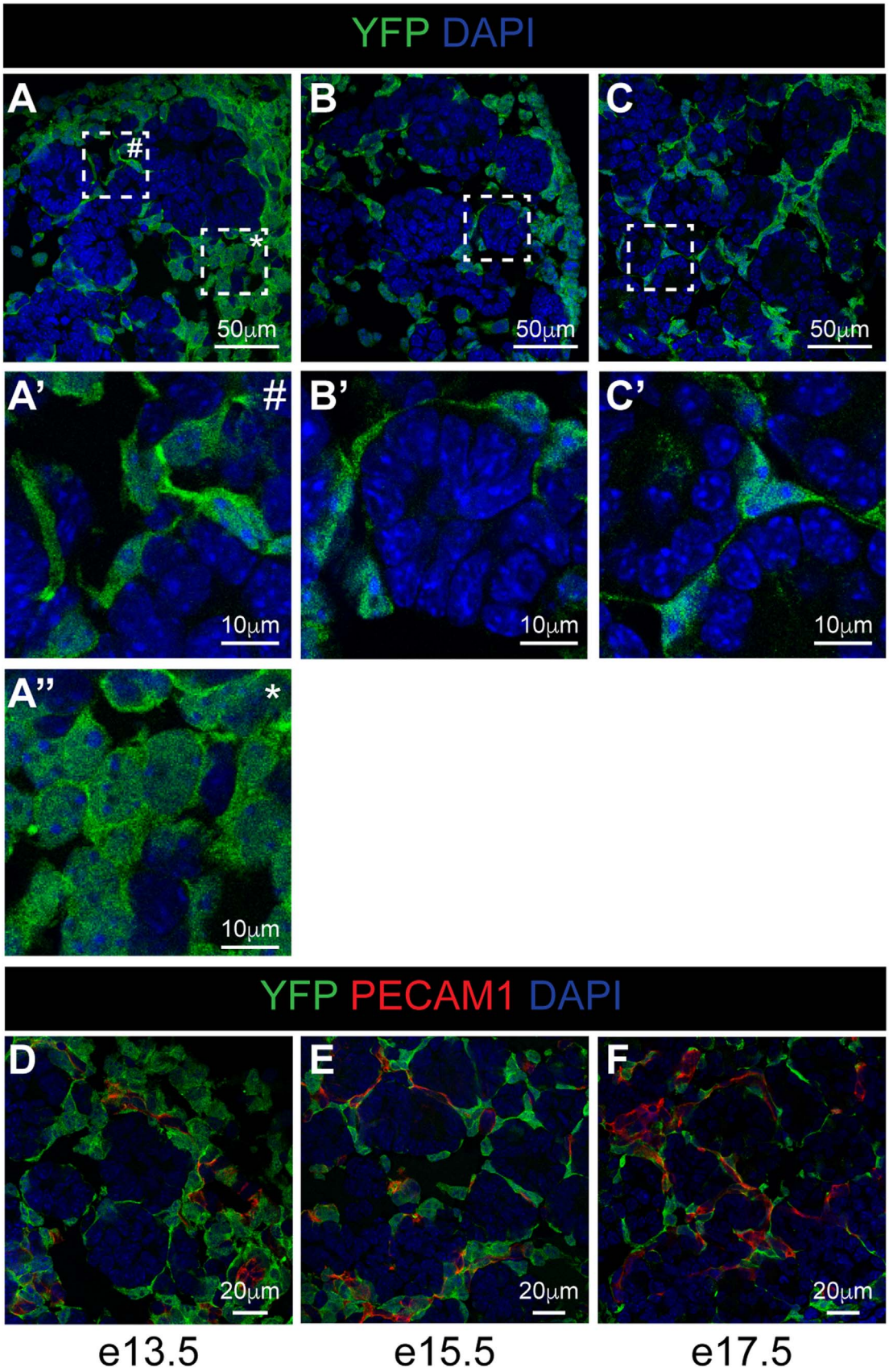

Fig. 4. Pancreatic mesenchymal cells display pericytic morphology and location. A –C) Immunofluorescence analysis of dissected pancreatic tissues from e13.5 (A), e15.5 (B), e17.5 (C) *Nkx3.2*-Cre;*LSL*-YFP embryos for YFP (green) and DAPI (blue). A′–C′ and A′′, Higher magnification of the areas framed in a white box. **D**–**F**) Immunofluorescence analysis of dissected pancreatic tissues from e13.5 (D), e15.5 (E), e17.5 (F) *Nkx3.2*-Cre;*LSL*-YFP embryos for YFP (green), the endothelial marker PECAM1 (red), and DAPI (blue).

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Declarations of interest

None.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2019.01.020.

References

Almaça, J., Weitz, J., Rodriguez-Diaz, R., Pereira, E., Caicedo, A., 2018. The pericyte of the pancreatic islet regulates capillary diameter and local blood flow. (e4)Cell Metab. 27, 630–644. http://dx.doi.org/10.1016/j.cmet.2018.02.016.

Angelo, J.R., Tremblay, K.D., 2018. Identification and fate mapping of the pancreatic mesenchyme. Dev. Biol. 435, 15–25. http://dx.doi.org/10.1016/j.ydbio.2018.01.003.

Armulik, A., Genové, G., Betsholtz, C., 2011. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev. Cell 21, 193–215. http://dx.doi.org/10.1016/j.devcel.2011.07.001.

Asahina, K., Zhou, Bin, Pu, W.T., Tsukamoto, H., 2011. Septum transversum-derived mesothelium gives rise to hepatic stellate cells and perivascular mesenchymal cells in developing mouse liver. Hepatology 53, 983–995. http://dx.doi.org/10.1002/hep.24119.

Byrnes, L.E., Wong, D.M., Subramaniam, M., Meyer, N.P., Gilchrist, C.L., Knox, S.M., Tward, A.D., Ye, C.J., Sneddon, J.B., 2018. Lineage dynamics of murine pancreatic development at single-cell resolution. Nat. Commun. 9, 3922. http://dx.doi.org/10.1038/s41467-018-06176-3.

Cheng Pan, F., Wright, C., 2011. Pancreas organogenesis: from bud to plexus to gland. Dev. Dyn. 240, 530–565. http://dx.doi.org/10.1002/dvdy.22584.

Dias Moura Prazeres, P.H., Sena, I.F.G., Borges, I.D.T., de Azevedo, P.O., Andreotti, J.P., de Paiva, A.E., de Almeida, V.M., de Paula Guerra, D.A., Pinheiro dos Santos, G.S., Mintz, A., Delbono, O., Birbrair, A., 2017. Pericytes are heterogeneous in their origin within the same tissue. Dev. Biol. 427, 6–11. http://dx.doi.org/10.1016/j.ydbio.2017.05.001.

Dreszer, T.R., Karolchik, D., Zweig, A.S., Hinrichs, A.S., Raney, B.J., Kuhn, R.M., Meyer, L.R., Wong, M., Sloan, C.A., Rosenbloom, K.R., Roe, G., Rhead, B., Pohl, A., Malladi, V.S., Li, C.H., Learned, K., Kirkup, V., Hsu, F., Harte, R.A., Guruvadoo, L., Goldman, M., Giardine, B.M., Fujita, P.A., Diekhans, M., Cline, M.S., Clawson, H., Barber, G.P., Haussler, D., James Kent, W., 2012. The UCSC Genome Browser database: extensions and updates 2011. Nucleic Acids Res. 40, D918–D923. http://dx.doi.org/10.1093/nar/gkr1055.

Epshtein, A., Rachi, E., Sakhneny, L., Mizrachi, S., Baer, D., Landsman, L., 2017a. Neonatal pancreatic pericytes support β-cell proliferation. Mol. Metab. 6, 1330–1338. http://dx.doi.org/10.1016/j.molmet.2017.07.010.

Epshtein, A., Sakhneny, L., Landsman, L., 2017b. Isolating and analyzing cells of the pancreas mesenchyme by flow cytometry. (e55344–e55344)JoVE. http://dx.doi.org/10.3791/55344.

Gittes, G.K., 2009. Developmental biology of the pancreas: a comprehensive review. Dev. Biol. 326, 4–35. http://dx.doi.org/10.1016/j.ydbio.2008.10.024.

Guo, T., Landsman, L., Li, N., Hebrok, M., 2013. Factors expressed by murine embryonic pancreatic mesenchyme enhance generation of insulin-producing cells from hESCs. Diabetes 62, 1581–1592. http://dx.doi.org/10.2337/db12-0167.

Hecksher-Sørensen, J., Watson, R.P., Lettice, L.A., Serup, P., Eley, L., De Angelis, C., Ahlgren, U., Hill, R.E., 2004. The splanchnic mesodermal plate directs spleen and pancreatic laterality, and is regulated by Bapx1/Nkx3.2. Development 131, 4665–4675. http://dx.doi.org/10.1242/dev.01364.

Houtz, J., Borden, P., Ceasrine, A., Minichiello, L., Kuruvilla, R., 2016. Neurotrophin signaling is required for glucose-induced insulin secretion. Dev. Cell 39, 329–345. http://dx.doi.org/10.1016/j.devcel.2016.10.003.

Landsman, L., Nijagal, A., Whitchurch, T.J., Vanderlaan, R.L., Zimmer, W.E., Mackenzie, T.C., Hebrok, M., 2011. Pancreatic mesenchyme regulates epithelial organogenesis throughout development. PLoS Biol. 9, e1001143. http://dx.doi.org/10.1371/journal.pbio.1001143.

Lettice, L., Hecksher-Sorensen, J., Hill, R., 2001. The role of Bapx1 (Nkx3.2) in the development and evolution of the axial skeleton. J. Anat. 199, 181–187.

Lewis, W.H., 1923. Mesenchyme and mesothelium. J. Exp. Med. 38, 257–262.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., 1000 Genome Project Data Processing Subgroup. 2009. The sequence alignment/Map format and SAMtoolsBioinformatics 25, 2078–2079. http://dx.doi.org/10.1093/bioinformatics/btp352.

Murgai, M., Ju, W., Eason, M., Kline, J., Beury, D.W., Kaczanowska, S., Miettinen, M.M., Kruhlak, M., Lei, H., Shern, J.F., Cherepanova, O.A., Owens, G.K., Kaplan, R.N., 2017. KLF4-dependent perivascular cell plasticity mediates pre-metastatic niche formation and metastasis. Nat. Med. 23, 1176–1190. http://dx.doi.org/10.1038/nm.4400.

Richards, O.C., Raines, S.M., Attie, A.D., 2010. The role of blood vessels, endothelial cells, and vascular pericytes in insulin secretion and peripheral insulin action. Endocr. Rev. 31, 343–363. http://dx.doi.org/10.1210/er.2009-0035.

Rinkevich, Y., Mori, T., Sahoo, D., Xu, P.-X., Bermingham, J.R., Weissman, I.L., 2012. Identification and prospective isolation of a mesothelial precursor lineage giving rise to smooth muscle cells and fibroblasts for mammalian internal organs, and their vasculature. Nat. Cell Biol. 14, 1251–1260. http://dx.doi.org/10.1038/ncb2610.

Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140. http://dx.doi.org/10.1093/bioinformatics/btp616.

Romer, A.I., Sussel, L., 2015. Pancreatic islet cell development and regeneration. Curr. Opin. Endocrinol. Diabetes Obes. 22, 255–264. http://dx.doi.org/10.1097/MED.0000000000000174.

Sakhneny, L., Khalifa-Malka, L., Landsman, L., 2018a. Pancreas organogenesis: approaches to elucidate the role of epithelial-mesenchymal interactions. Semin. Cell Dev. Biol.. http://dx.doi.org/10.1016/j.semcdb.2018.08.012.

Sakhneny, L., Rachi, E., Epshtein, A., Guez, H.C., Wald-Altman, S., Lisnyansky, M., Khalifa-Malka, L., Hazan, A., Baer, D., Priel, A., Weil, M., Landsman, L., 2018b. Pancreatic pericytes support β-cell function in a Tcf7l2-dependent manner. Diabetes 67, 437–447. http://dx.doi.org/10.2337/db17-0697.

Sasson, A., Rachi, E., Sakhneny, L., Baer, D., Lisnyansky, M., Epshtein, A., Landsman, L., 2016. Islet pericytes are required for β-cell maturity. Diabetes 65, 3008–3014. http://dx.doi.org/10.2337/db16-0365.

Song, S., Ewald, A.J., Stallcup, W., Werb, Z., Bergers, G., 2005. PDGFR|[beta]|+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. Nat. Cell Biol. 7, 870–879. http://dx.doi.org/10.1038/ncb1288.

Tang, S.-C., Chiu, Y.-C., Hsu, C.-T., Peng, S.-J., Fu, Y.-Y., 2013. Plasticity of Schwann cells and pericytes in response to islet injury in mice. Diabetologia 56, 2424–2434. http://dx.doi.org/10.1007/s00125-013-2977-y.

Trapnell, C., Pachter, L., Salzberg, S.L., 2009. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25, 1105–1111. http://dx.doi.org/10.1093/bioinformatics/btp120.

Verzi, M.P., Stanfel, M.N., Moses, K.A., Kim, B.-M., Zhang, Y., Schwartz, R.J., Shivdasani, R.A., Zimmer, W.E., 2009. Role of the homeodomain transcription factor Bapx1 in mouse distal stomach development. Gastroenterology 136, 1701–1710. http://dx.doi.org/10.1053/j.gastro.2009.01.009.

Wells, J.M., Melton, D.A., 1999. Vertebrate endoderm development. Annu. Rev. Cell Dev. Biol. 15, 393–410. http://dx.doi.org/10.1146/annurev.cellbio.15.1.393.

Wilm, B., Ipenberg, A., Hastie, N.D., Burch, J.B.E., Bader, D.M., 2005. The serosal mesothelium is a major source of smooth muscle cells of the gut vasculature. Development 132, 5317–5328. http://dx.doi.org/10.1242/dev.02141.

Winkler, E.A., Bell, R.D., Zlokovic, B.V., 2011. Central nervous system pericytes in health and disease. Nat. Neurosci. 14, 1398–1405. http://dx.doi.org/10.1038/nn.2946.

Yamazaki, T., Nalbandian, A., Uchida, Y., Li, W., Arnold, T.D., Kubota, Y., Yamamoto, S., Ema, M., Mukouyama, Y.-S., 2017. Tissue myeloid progenitors differentiate into pericytes through TGF-β signaling in developing skin vasculature. Cell Rep. 18, 2991–3004. http://dx.doi.org/10.1016/j.celrep.2017.02.069.