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Duct cells in development, regeneration, and transplantation: charting a path to new islets

Balak, J.R.A.

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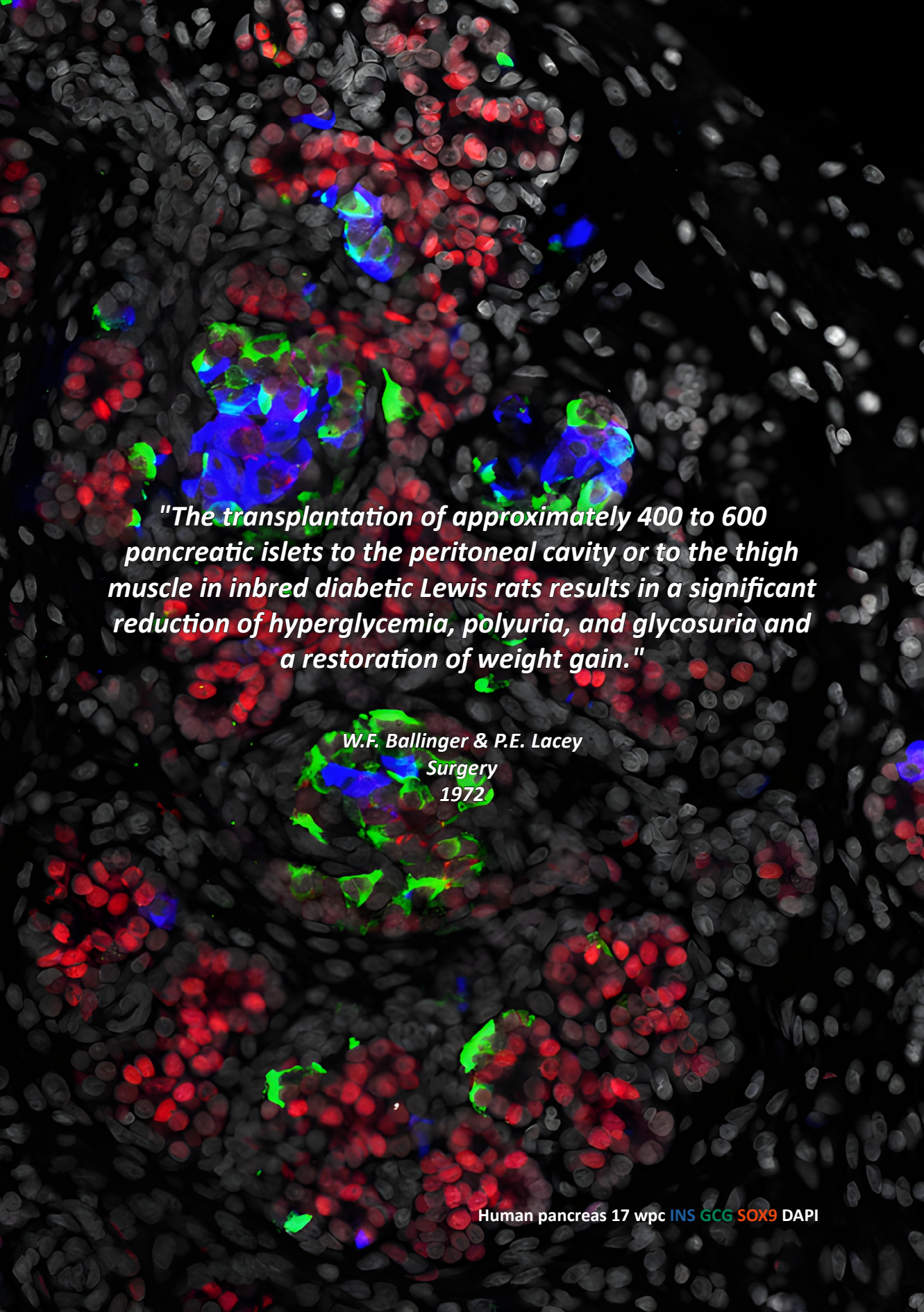
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"The transplantation of approximately 400 to 600 pancreatic islets to the peritoneal cavity or to the thigh muscle in inbred diabetic Lewis rats results in a significant reduction of hyperglycemia, polyuria, and glycosuria and a restoration of weight gain."

W.F. Ballinger & P.E. Lacey
Surgery
1972

Human pancreas 17 wpc INS GCG SOX9 DAPI

CHAPTER| 7

Cytoplasmic SOX9 Expression in Human Pancreas Development

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In preparation

Abstract

Deciphering the transcriptional regulation of human pancreatic islet development will facilitate the generation of novel regenerative strategies for the treatment of diabetes mellitus. Recently, a proliferative subset of adult human alpha cells was identified displaying cytoplasmic expression of SOX9 (SOX9^{cyto}), a transcription factor typically localized to the nucleus of pancreatic progenitor cells during development and to exocrine duct cells in the adult pancreas. Nucleocytoplasmic shuttling of SOX9 is an essential mechanism by which SOX9 regulates cell fate, but its potential implication in human pancreas development is unknown. We performed histological assessment of first and second trimester human pancreatic samples. We confirmed the expression of SOX9^{cyto} restricted to a subset of alpha cells in the adult human pancreas. In contrast, we identified a subpopulation of SOX9^{cyto} cells among fetal beta cells located both in the ductal epithelium and in endocrine cell clusters, and present throughout the development. These findings suggest that nucleocytoplasmic shuttling of SOX9 might play a role in islet cell specification during human pancreas development. This knowledge may ultimately contribute to further optimisation of strategies for beta cell replacement therapy from alternative cell sources.

Introduction

An incomplete understanding of the transcriptional regulation of human islet development hampers the formation of fully functional beta cells from pluripotent stem cells¹. Recent investigations initially focused on finding the origin of residual beta cells in T1D individuals, led to the identification of a highly proliferative islet cell subset in perinatal, adolescent, and young adult pancreata². Islet cell proliferation was comparable in healthy and T1D individuals, suggesting a normal physiological process involved in islet cell plasticity rather than a regenerative response to beta cell deficiency. The proliferating islet cells expressed alpha cell markers (GCG, ARX) together with SOX9, that was unexpectedly expressed in the cytoplasm.

The nuclear expression of SOX9 in pancreatic progenitor cells is required for maintenance of pancreatic progenitor cells, but also for endocrine cell formation³. Individuals affected by the lethal SOX9 haploinsufficiency syndrome campomelic hypoplasia show endocrine hypoplasia⁴, and transgenic rodent models show that SOX9 haploinsufficiency results in decreased number of NEUROG3-positive cells and islet hypoplasia⁵.

Sex-determining region Y (SRY)-box 9 protein (SOX9) is a member of the SOX family of transcription factors, which are developmental regulators defined by the presence of a highly conserved high-mobility group (HMG) domain that mediates DNA binding⁶. These unique binding properties, in addition to other complex regulatory mechanisms at the level of transcription, translation, post-translation modifications, and cofactor interaction, enables highly dynamic, flexible, and accurately controlled expression of SOX9, resulting in stage-specific functions in a multitude of tissues and cells⁶. The access of SOX proteins to the nucleus via nucleocytoplasmic shuttling is known to be a key driver of developmental switches and programmed cell differentiation, and is

regulated by posttranslational modifications such as acetylation, phosphorylation, sumoylation, and ubiquitination^{7,8}.

Although the requirement of nuclear SOX9 for normal pancreas organogenesis and endocrine development has been well established, the presence of SOX9^{cyto} in human pancreas development has not been described. Here we report the identification of a subset of human fetal beta cells displaying the cytoplasmic expression of SOX9, which - given the versatile function of SOX9 - might play an important process by which endocrine cell specification is regulated in the pancreas.

Materials and methods

Human fetal and adult pancreas collection

The collection and use of fetal material were carried out following ethical approval from the Medical Ethics committee in the LUMC. Human fetal tissue ranging from 7 to 20 wpc (weeks post conception) (n=14) was collected with informed consent after termination of pregnancy performed by vacuum aspiration in an elective abortion clinic. Human adult pancreatic tissue was derived from the clinical islet transplantation program at the Leiden University Medical Center and was only used when research consent was present.

Immunohistochemistry and microscopy

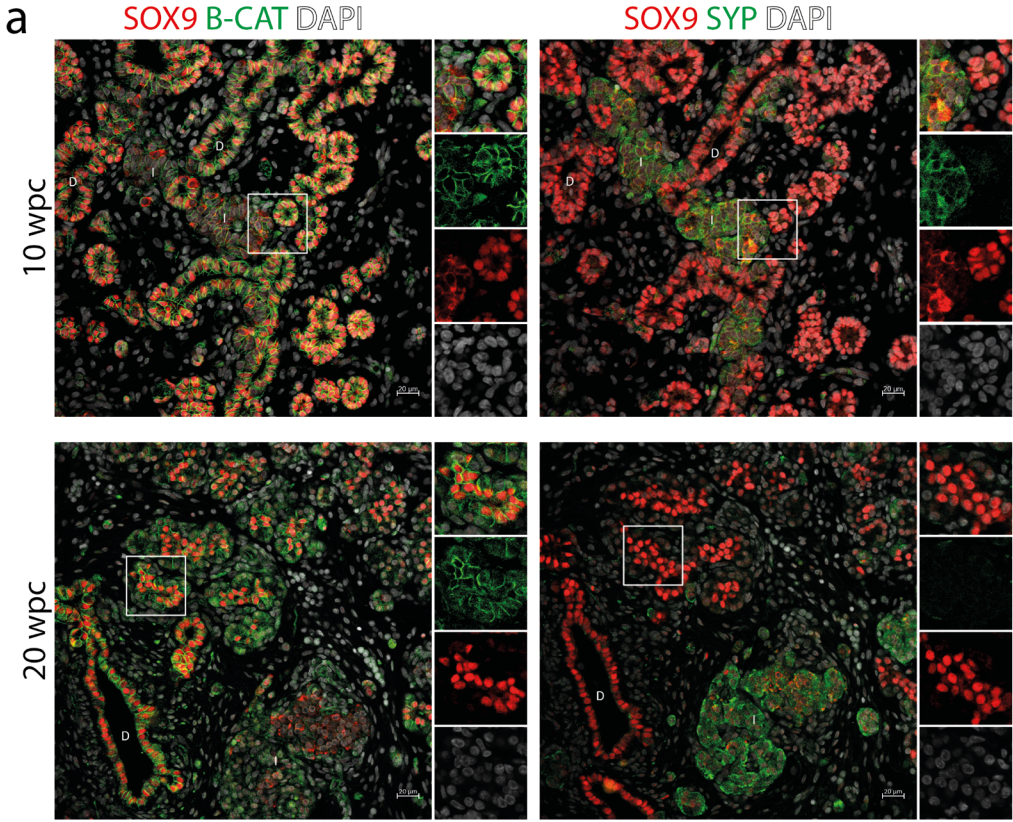
Tissue was processed and sectioned as previously described⁹. Heat-induced epitope retrieval was performed with a pressure cooker using a citrate buffer (0.01 M citric acid, pH 6.0). After blocking with normal donkey serum, slides were incubated with the primary antibodies for one hour at room temperature or overnight at 4 °C, for immunofluorescent detection slides were incubated for one hour with an appropriate secondary antibody (**Supplemental Table 1**). ARX was visualized using a Tyramide Signal Amplification set according to manufacturer's protocol (Perkin Elmer). Nuclei were counterstained with DAPI (Vector laboratories). Images were recorded using an LSM 7 MP confocal microscope (Zeiss) and were processed using the Zen Lite software (Zeiss). Quantification was performed by point counting positive cells by hand using ImageJ (National Institute of Health). A minimum of 1500 cells were quantified per fetal donor (average of 4300 cells/donor).

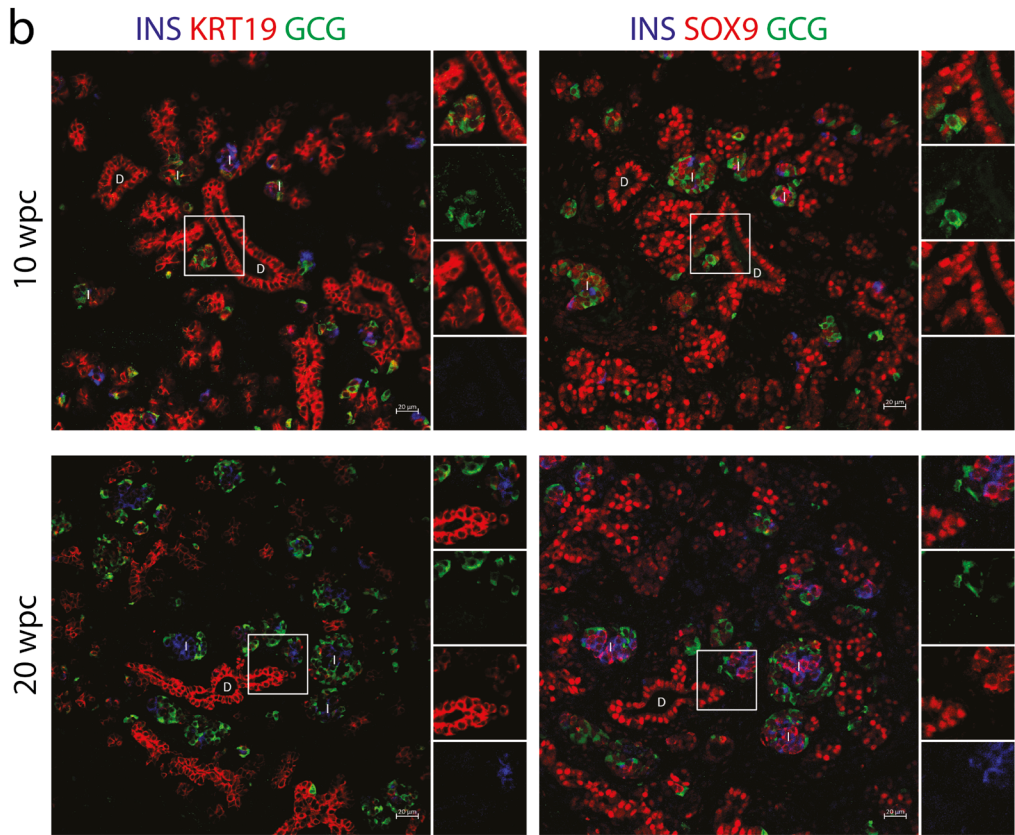
Results

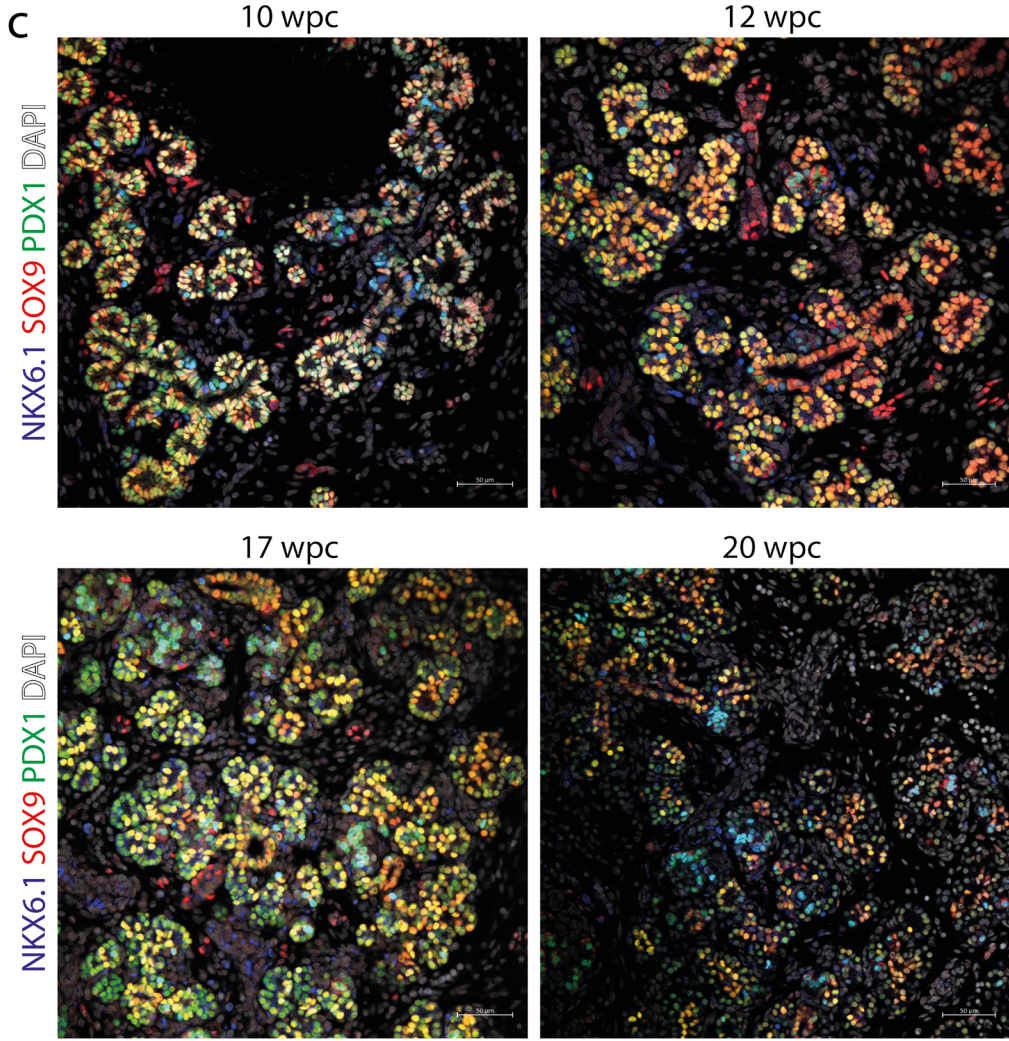
SOX9^{cyto} is expressed in the human pancreas during development

Differentiated structures such as ducts and endocrine cell clusters could be identified in first and second trimester samples using β -catenin (epithelial marker), keratin 19 (duct marker), SOX9 (pancreatic progenitor and duct marker) and synaptophysin (pan-endocrine marker) (**Figure 1a-b**). Faint KRT19 expression was observed in endocrine cell clusters in early fetal samples and decreased with advancing gestational age, as previously described^{10,11}. Nuclear SOX9 was abundantly co-expressed with PDX1 in the duct epithelium throughout development (**Figure 1c**)⁴. In order

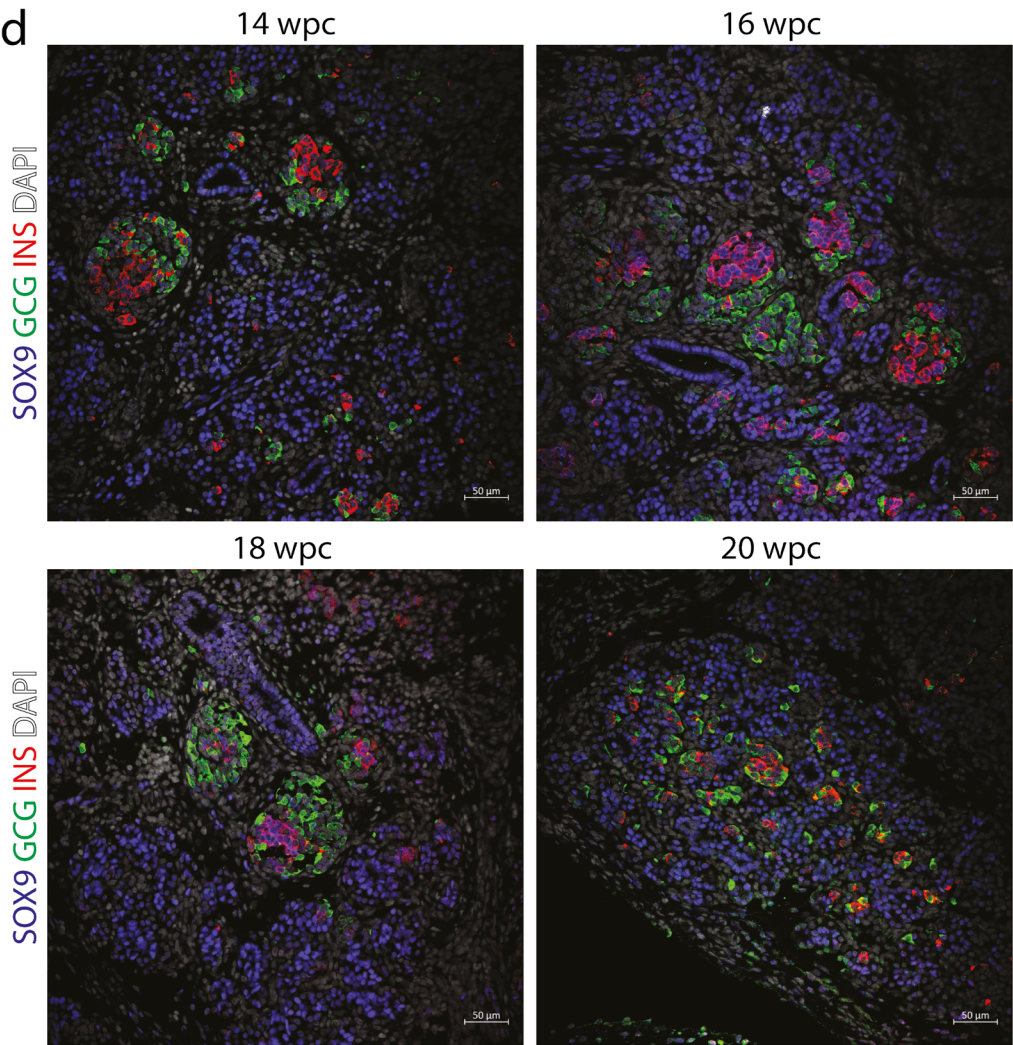
to examine SOX9 subcellular expression, specifically in the fetal endocrine compartment, we performed co-immunostainings for SOX9 and synaptophysin (**Figure 1a**) or insulin and glucagon (**Figure 1d**). Strikingly, SOX9^{cyto}-positive cells were found in and around the endocrine cell clusters, showing that SOX9^{cyto} is expressed in a subset of endocrine cells in human pancreatic fetal tissue.



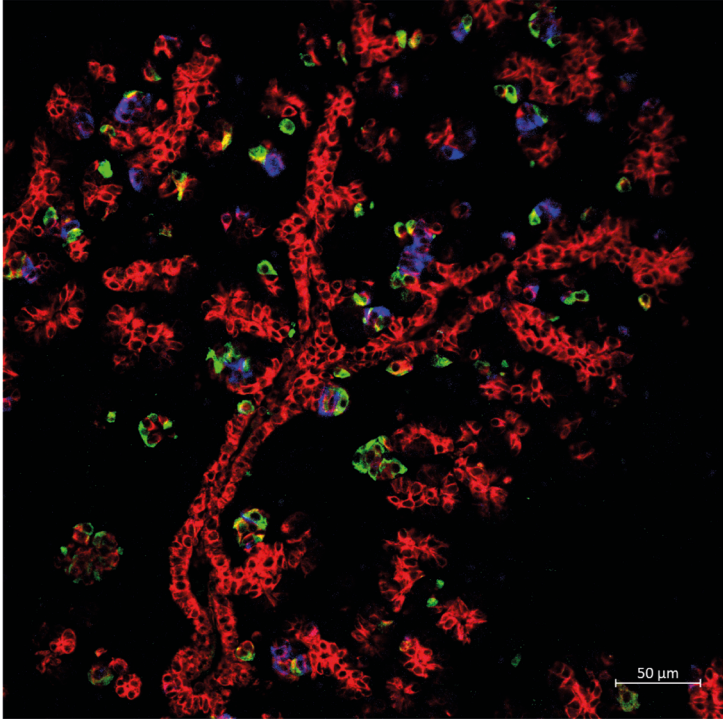




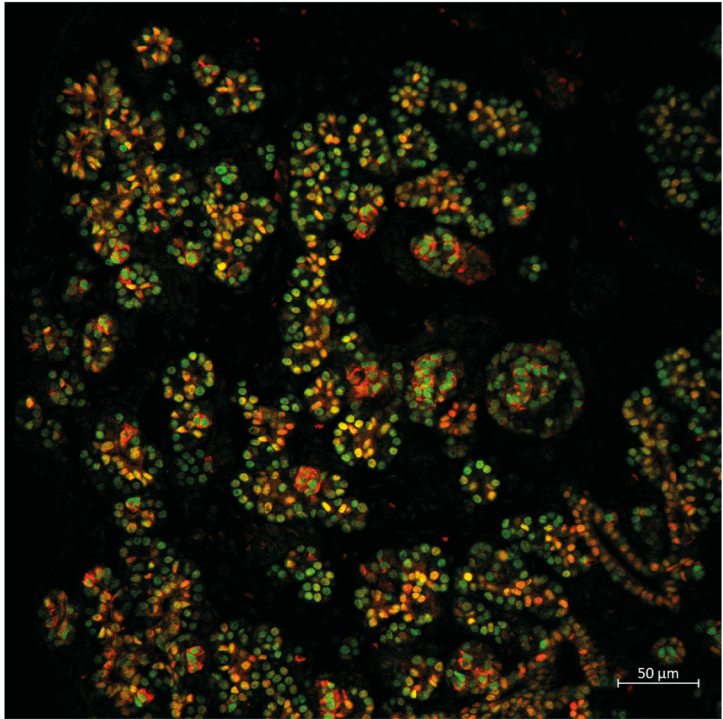
d



e 10 wpc INS KRT19 GCG



f 10 wpc SOX9 PDX1



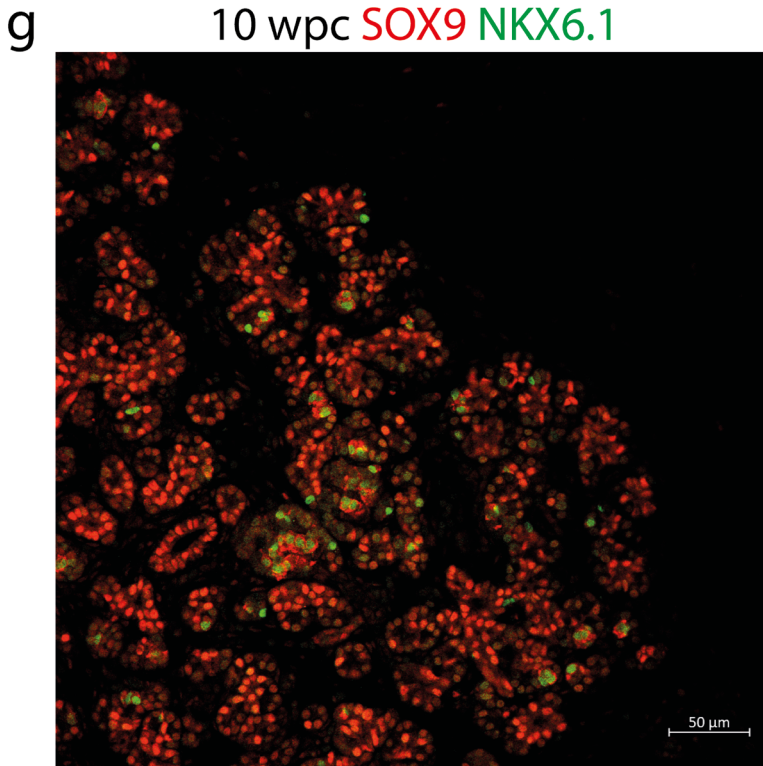


Figure 1. Identification of differentiated pancreatic structures during human development

(a) Representative sections of human fetal tissue of 10 wpc and 20 wpc with immunofluorescent staining for β -CAT (β -catenin, epithelial marker, green), SOX9 (ductal marker, red); and SYP (synaptophysin, endocrine cell marker, green), SOX9 (red), showing bright expression of β -catenin in ductal cells. The fetal endocrine cell clusters have a faint expression of β -catenin and also express synaptophysin. D=duct, I= islet. Scale bar = 20 μ m. (b) Immunofluorescent staining for GCG (glucagon, alpha cell marker, green), KRT19 (keratin 19, ductal marker, red), INS (insulin, beta cell marker) showing expression of KRT19 in the pancreatic duct and a faint staining in the endocrine cell clusters. D=duct, I= islet. Scale bar = 20 μ m. (c) Sections of human fetal tissue of 10 wpc, 12 wpc, 17 wpc and 20 wpc with immunofluorescent staining for NKX6.1 (beta cells marker, blue), SOX9 (ductal marker, red), PDX1 (beta cell marker, green) showing expression of NKX6.1 /PDX1-double-positive cells in the ductal epithelium and fetal islets throughout gestation. Scale bar = 50 μ m. (d) Sections of human fetal tissue of 14 wpc, 16 wpc, 18 wpc and 20 wpc with immunofluorescent staining for SOX9 (duct marker, blue), GCG (glucagon, alpha cell marker, green), INS (insulin, beta cell marker, red) showing fetal endocrine cells in islets, also expressing cytoplasmic SOX9. Scale bar = 50 μ m. (e-g) Human fetal tissue of 10 wpc with immunofluorescent staining for INS (insulin, beta cell marker, blue), KRT19 (keratin 19, duct cell marker, red), GCG (glucagon, alpha cell marker, green); SOX9 (duct marker, red), PDX1 (beta cell marker, green); and SOX9 (duct cell marker, red) and NKX6.1 (beta cell marker, green), showing the branching ductal epithelium and developing islets. Scale bar = 50 μ m.

Proliferating fetal pancreatic cells rarely express SOX9^{cyto}

We next assessed whether cytoplasmic expression of SOX9 was related to proliferation, as reported

for the subset of islet cells identified in the early postnatal and adult pancreas². We performed co-immunostainings for Ki67 and SOX9 in samples of a 17 wpc pancreas, and found that the majority of proliferating cells were in the mesenchyme and pancreatic ducts, while only sporadic SOX9^{cyto} cells expressed Ki67 (**Supplemental Figure 1**). This indicates that SOX9^{cyto}-positive cells do not represent a proliferative cell population during pancreatic development.

SOX9^{cyto} is expressed in endocrine cells during human pancreas development

We next examined which endocrine cell type predominantly expressed SOX9^{cyto}, focusing on alpha and beta cells, as these contribute most to the endocrine compartment during human pancreatic development and in the postnatal pancreas (**Figure 2a**)^{10,12,13}. The fraction of SOX9^{cyto}-positive cells relative to the total number of SOX9-positive cells (SOX9^{nuc} and SOX9^{cyto}) increased with gestational age (**Figure 2b**), reaching up to 13% in the second trimester samples. We found SOX9^{cyto} to be expressed mainly in insulin-positive cells: at 10 wpc, 25% of SOX9^{cyto}-positive cells expressed insulin and the fraction of cells co-expressing insulin and glucagon was 29% (**Figure 2c**), while only 4% of SOX9^{cyto}-positive cells expressed glucagon only. Polyhormonal cells have been described both in immature endocrine cells during early human pancreatic development and in pluripotent stem cell-derived endocrine cells differentiated *in vitro*¹⁴⁻¹⁸. The proportion of polyhormonal SOX9^{cyto}-positive cells declined during development, decreasing to 11% between 13 and 18 wpc, and further dropping to 2% by 20 wpc. This reduction was accompanied by a concomitant increase in SOX9^{cyto}-expression in insulin-only-expressing cells, with 65% of SOX9^{cyto}-positive cells co-expressing insulin at 14 wpc. Throughout development, we observed a fraction of SOX9^{cyto}-positive cells negative for both insulin and glucagon, which ranged from 41% at 13 wpc, decreased to 15% at 14 wpc, and then increased up to 39% at later gestational ages.

We next evaluated the distribution of SOX9-positive cells in the insulin-positive cell population, and found an average of 21% of insulin-positive cells expressed SOX9^{cyto} throughout pancreas development (**Figure 2d**). In contrast, only 8% of glucagon-positive cells were SOX9-positive, excluding the 10 wpc sample, which contained a high fraction of insulin/glucagon-double-positive cells (**Figure 2a**). From these SOX9-positive insulin or glucagon-positive cells, the large majority of the cells expressed SOX9 in the cytoplasm. Yet, in the early gestational age samples we also observed some hormone-positive cells with SOX9 expression in the nuclear, possibly representing cells in transition from duct to endocrine cells (**Figure 2d-e**).

Altogether, our data demonstrate that the cytoplasmic expression of SOX9 is present in endocrine cells during fetal development and the majority of these cells are polyhormonal-positive for insulin and glucagon at early gestational age, and monohormonal insulin-positive at a later gestational age. This is in contrast with postnatal pancreatic cells, where cytoplasmic SOX9 expression was observed exclusively in a subset of alpha-like cells (**Figure 2a 'Adult'**)².

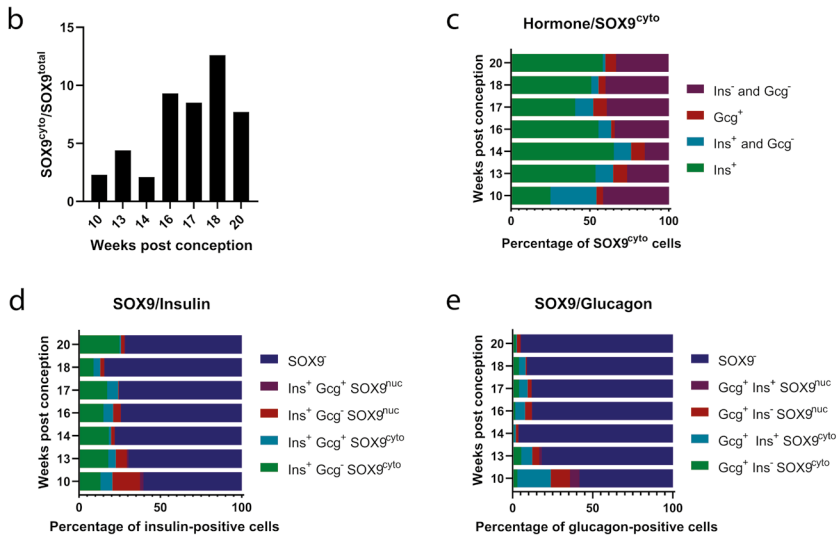


Figure 2. The cytoplasmic expression of SOX9 in hormone-positive cells during human pancreas development

(a) Immunofluorescent staining of human fetal pancreatic tissue for INS (insulin, beta cell marker, blue), SOX9 (ductal marker, red), GCG (glucagon; alpha cell marker, green), DAPI (nuclear marker, white), showing the cytoplasmic expression of INS/GCG-double-positive cells at 10 wpc and subsequently INS-positive cells at 13, 17 and 20 wpc. In human adult pancreatic tissue cytoplasmic expression of SOX9 is restricted to GCG-positive cells. Arrowheads point to SOX9^{cyto}-positive cells. Scale bar = 20 μ m. (b) Quantification of immunofluorescent stainings for INS/GCG/SOX9 showing the fraction SOX9^{cyto} of SOX9^{total} (SOX9^{nuc} and SOX9^{cyto}) cells in human fetal pancreatic tissue, showing an increase in the fraction SOX9^{cyto}/SOX9^{total} in second trimester samples. (c) Quantification of immunofluorescent stainings for INS/GCG/SOX9 showing SOX9^{cyto} subpopulations, SOX9^{cyto} cells mainly colocalize with insulin-positive cells. In the first trimester there is a large fraction of polyhormonal cells observed, which decreased in the second trimester. (d) Quantification of immunofluorescent stainings for INS/GCG/SOX9 showing the fraction of SOX9^{cyto} cells in the total insulin-positive cell population, the SOX9^{cyto}-negative cell fraction of insulin-positive cells is stable around 73% throughout development. (e) Quantification of immunofluorescent stainings for INS/GCG/SOX9 showing the fraction of SOX9^{cyto}-positive cells in total glucagon-positive cell population, the percentage of SOX9^{cyto}-negative cells is around 90% during development, with the exception of 12 wpc due to a high fraction of polyhormonal cells.

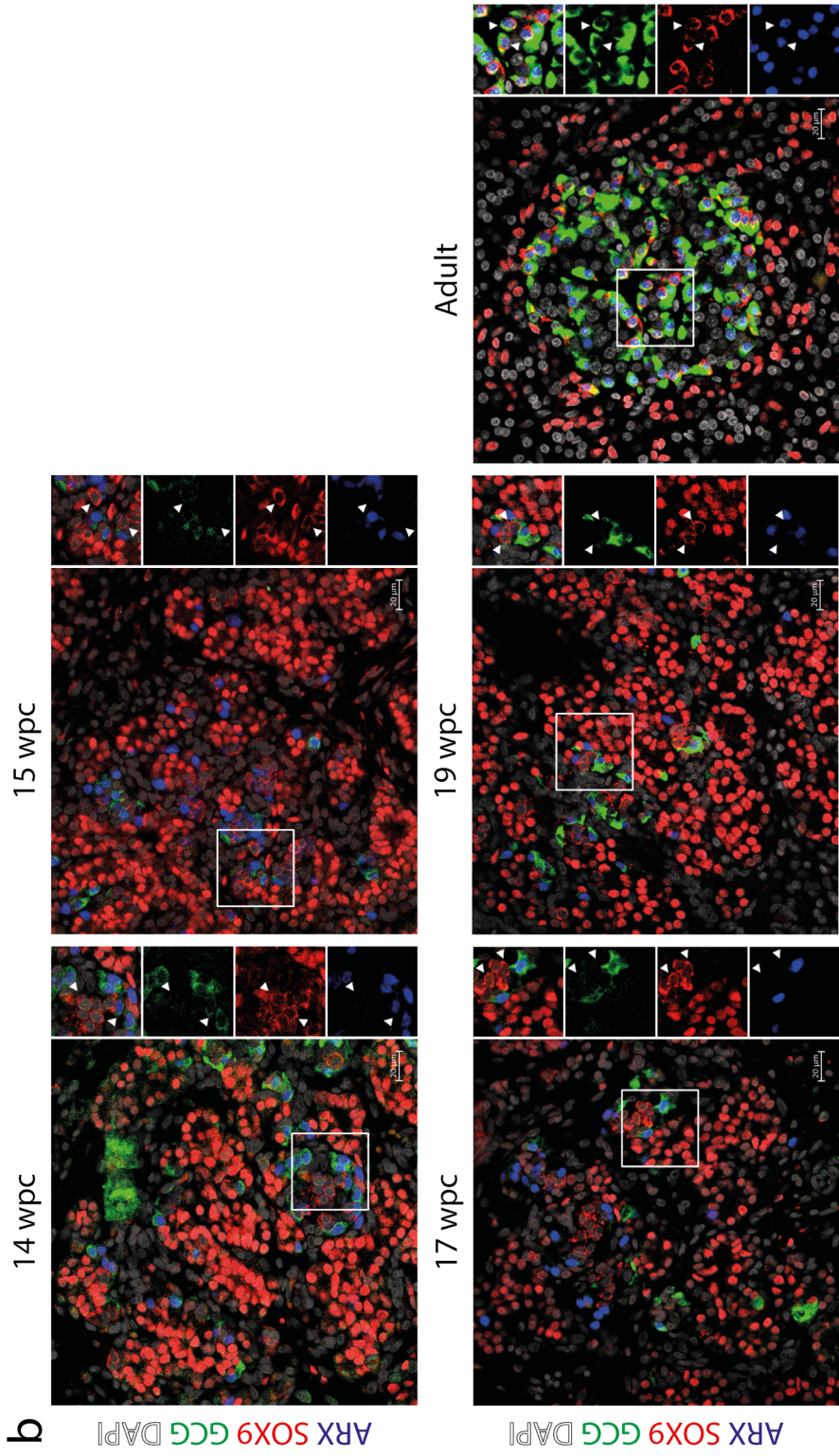
SOX9^{cyto} is expressed in a subset of fetal beta cells

We next wondered whether cytoplasmic expression of SOX9 could play a role in endocrine cell specification in pancreatic progenitor cells (Figure 3a, Figure 3c-g). At 6 wpc, pancreatic progenitor cells start to organize in a tip-trunk segregation, after which the endocrine cells will form from SOX9/PDX1-double-positive trunk cells, whereas PDX1/CPA-double-positive tip cells will form exocrine cells¹⁹. The percentage of SOX9^{nuc}-positive cells expressing PDX1 was 60-80% (Figure 3c). The SOX9^{cyto}-positive cell population was composed for more than 50% of PDX1 and/or NKX6.1-

positive cells at all gestational ages (**Figure 3d**). At 10 wpc, 33% of SOX9^{cyto} cells were PDX1-positive and NKX6.1-negative, whereas 11% of cells was double-positive for PDX1 and NKX6.1. During the later stages of development, the majority of SOX9^{cyto} cells was double-positive for NKX6.1 and PDX1, reaching up to 47% cells in the later gestational ages.

The percentage of SOX9^{cyto}-positive cells of all PDX1-positive cells increased during gestation up to 8% at 18 wpc (**Figure 3e**). The majority of these cells was also positive for NKX6.1. For the NKX6.1-positive cell population, only 1% of NKX6.1-positive cells was SOX9^{cyto}-positive at 10 wpc, increasing to 50% at 18 wpc (**Figure 3f**). More than 90% of these cells co-expressed PDX1. When looking at the total amount of NKX6.1/PDX1-double-positive cells, more than half was SOX9^{cyto}-positive with the exception at 10 wpc (**Figure 3g**).

Because SOX9^{cyto} in the postnatal pancreas is mostly expressed in a subset of alpha-like cells, we also performed co-immunostainings with the alpha cell lineage-specific transcription factor ARX. The expression of ARX was observed in cells expressing glucagon, however we rarely found ARX-cells co-expressing SOX9^{cyto} (**Figure 3b, Figure 3h**). In contrast, in adult tissue we found ARX-positive cells co-expressing glucagon and SOX9^{cyto}, as previously reported (**Figure 3b 'Adult'**)².



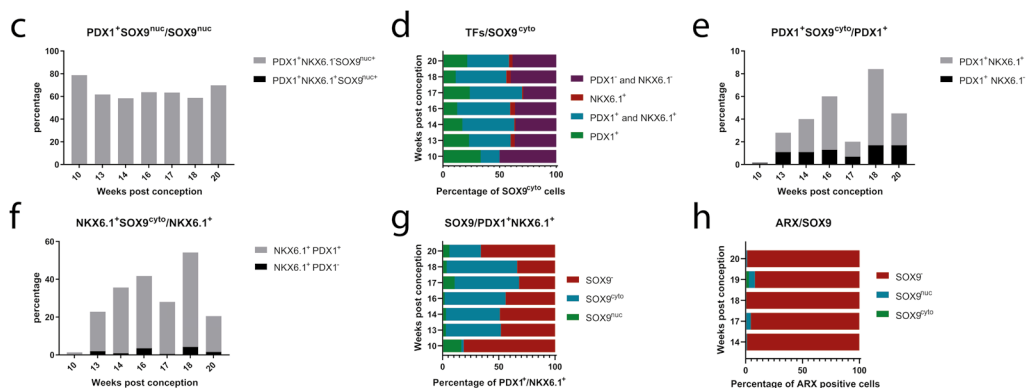


Figure 3. SOX9^{cyto} is expressed in cells expressing only beta cell markers in fetal tissue, but restricted to cells with only alpha cell markers in adult tissue

(a) Immunofluorescent staining of human fetal pancreatic tissue for PDX1 (beta cell marker, blue), SOX9 (ductal marker, red), NKX6.1 (beta cell marker, green), DAPI (nuclear marker, white), showing SOX9^{cyto} expression in PDX1/NKX6.1-positive cells throughout development. In human adult pancreatic islets SOX9^{cyto} expression is restricted to PDX1/NKX6.1-double negative cells. Arrowheads point to SOX9^{cyto}-positive cells. Scale bar = 20 μ m. (b) Immunofluorescent staining of human fetal pancreatic tissue for ARX (alpha cell marker, blue), SOX9 (ductal marker, red), GCG (alpha cell marker, green), DAPI (nuclear marker, white), showing islets with SOX9^{cyto}-expression in ARX/GCG-double negative cells throughout development. In adult tissue SOX9^{cyto} is expressed in ARX/GCG-double-positive cells. Arrowheads point to SOX9^{cyto}-positive cells. Scale bar = 20 μ m. (c) Quantification of immunofluorescent stainings for PDX1/NKX6.1/SOX9, showing that the percentage of PDX1/SOX9^{nuc}-double-positive cells of the SOX9^{nuc}-population is between 60-80% during development. The fraction of PDX1/SOX9^{nuc}-double cells also positive for NKX6.1 is <1%. (d) Quantification of immunofluorescent stainings for PDX1/NKX6.1/SOX9, indicating that throughout development 50-70% of SOX9^{cyto} cells is positive for PDX1 and/or NKX6.1. (e) Quantification of immunofluorescent stainings for PDX1/NKX6.1/SOX9, showing that the percentage of SOX9^{cyto}-positive cells in the PDX1-positive population increased during gestation, up to 8% at 18 wpc. The majority of PDX1/SOX9^{cyto}-double-positive cells also express NKX6.1. (f) Quantification of immunofluorescent stainings for PDX1/NKX6.1/SOX9 showing the percentage of SOX9^{cyto}-positive cells in the NKX6.1-positive population. There is a large increase in the percentage of SOX9^{cyto}-positive cells during development, at 18 wpc almost 55% of NKX6.1- positive cells expresses SOX9^{cyto}, and almost all cells also co-express PDX1. (g) Quantification of immunofluorescent stainings for PDX1/NKX6.1/SOX9, showing there is a large increase of SOX9^{cyto} cells in the PDX1/NKX6.1-double-positive population during development. (h) Quantification of immunostainings for ARX/SOX9/GCG, indicating that <1% of ARX-positive cells expressed SOX9^{cyto}.

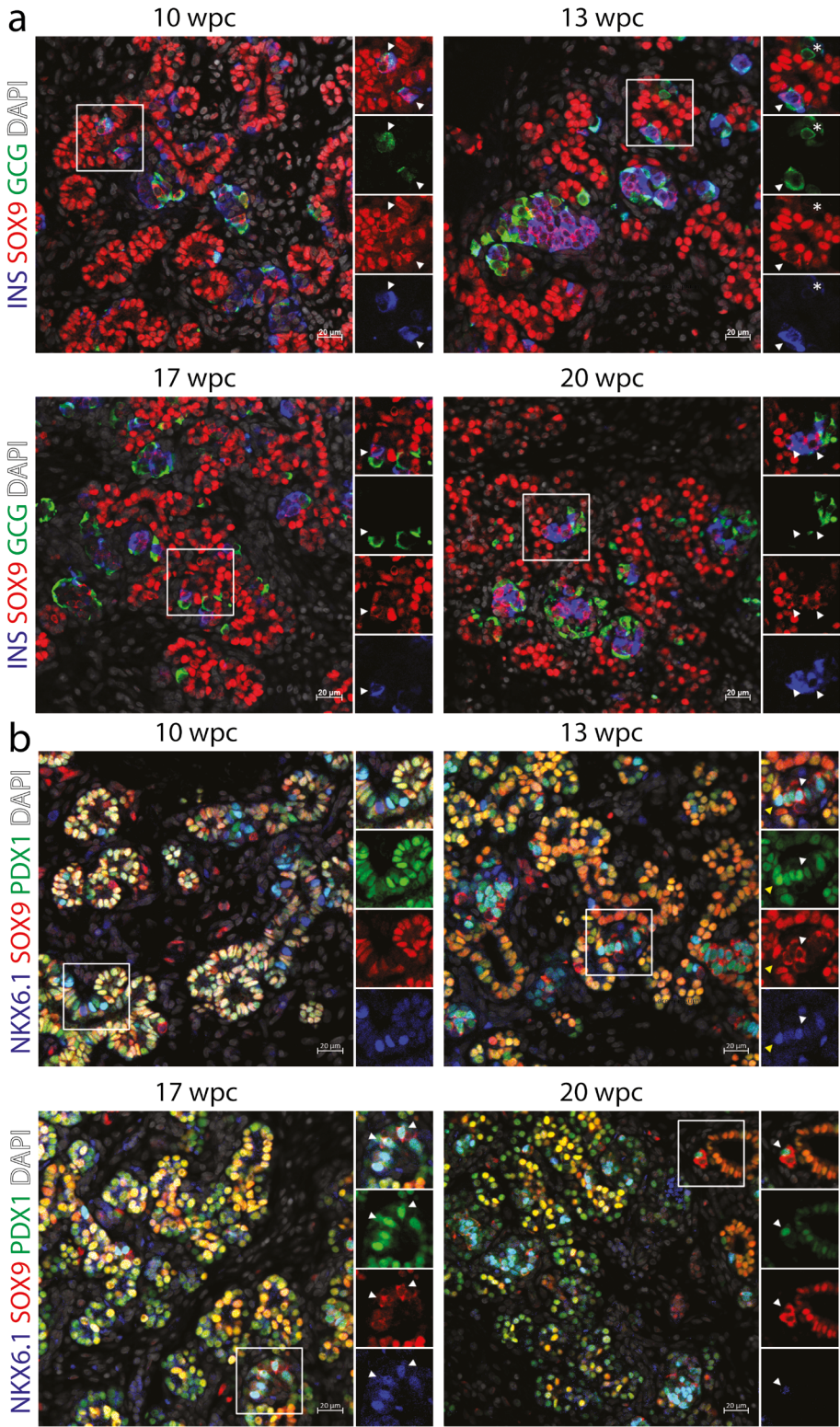
SOX9^{cyto}-positive cells are located in endocrine cell clusters as well as the ductal epithelium

To evaluate whether SOX9 is expressed in the cytoplasm of progenitor cells before differentiation, delamination, and migration into the endocrine cell clusters, we examined the location of SOX9^{cyto}-positive cells. We identified insulin/SOX9^{cyto}-double-positive cells and PDX1/NKX6.1/SOX9^{cyto}-

triple-positive cells in and around the ductal epithelium, as well as in endocrine cell clusters (**Figure 4a-b**). In addition, we observed SOX9^{cyto}-negative/insulin-positive cells, suggesting a transient SOX9^{cyto} expression and heterogeneity in the beta cell and beta cell progenitor population.

We further examined the NKX6.1-positive cell population. While this marker can be indicative for pancreatic endocrine progenitors²⁰, we found that almost all NKX6.1/SOX9^{cyto}-double-positive cells were also insulin-positive, indicative of a beta cell phenotype instead (**Figure 4c**). Of note, we also identified a subpopulation of PDX1/NKX6.1^{bright}-double-positive cells in the ducts that were insulin/SOX9-double-negative (**Figure 4c**). Furthermore, we observed SOX9^{cyto}/glucagon-positive cells located in and around a large duct in the adult pancreas (**Figure 4d**).

In conclusion, a subset of fetal beta cells positive for insulin, PDX1 and NKX6.1 express SOX9^{cyto}, and although the majority of cells can be found in the endocrine cell clusters there are also sporadic cells in the ductal epithelium lining. In adult tissue SOX9^{cyto} can be found in duct structures co-localizing with glucagon.



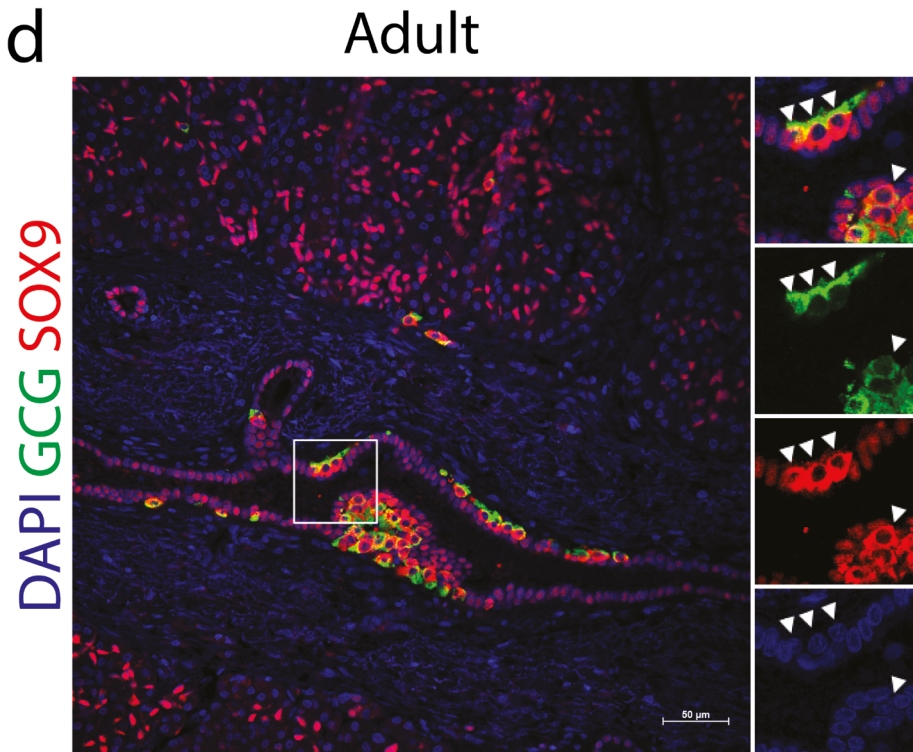
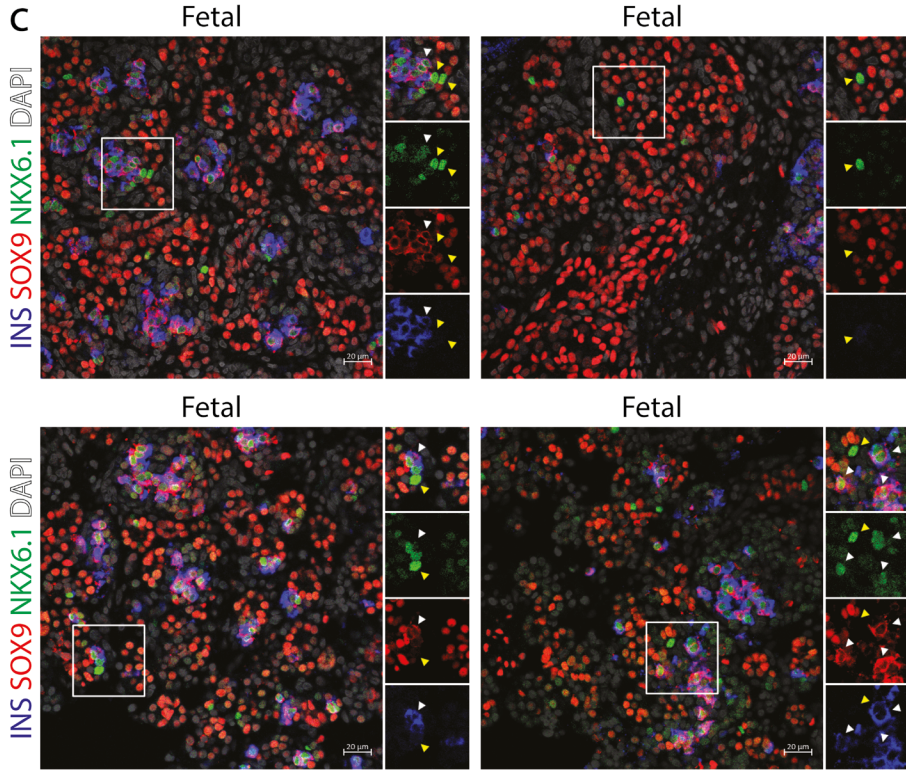


Figure 4. SOX9^{cyto}-positive cells in human fetal pancreatic tissue are located in endocrine cell clusters as well as ductal structures

(a) Immunofluorescent staining for INS (insulin, beta cell marker, blue), SOX9 (ductal cell marker, red) and GCG (glucagon, alpha cell marker, green). White arrows point to hormone-positive cells located in or near the ductal epithelium that are also SOX9^{cyto} positive. The asterisk indicates a glucagon-positive cell that is insulin and SOX9 negative. Scale bar = 20 μ m. (b) Immunofluorescent staining for PDX1 (beta cell marker, blue), SOX9 (ductal marker, red), and NKX6.1 (beta cell marker, green). White arrowheads point to PDX1/NKX6.1-double-positive cells in the ductal epithelium that are also positive for cytoplasmic SOX9, yellow arrowheads points to PDX1/NKX6.1-double-positive cells in the ductal epithelium that are SOX9^{cyto} negative. Scale bar = 20 μ m. (c) Immunofluorescent staining for INS (insulin, beta cell marker, blue), SOX9 (ductal cell marker, red) and NKX6.1 (beta cell marker, green). White arrowheads point to insulin-positive cells with a faint NKX6.1-expression that are also SOX9^{cyto}-positive. Yellow arrowheads point to NKX6.1^{bright}-positive cells that are insulin and SOX9-negative. Scale bar = 20 μ m. (d) Immunofluorescent staining of human pancreatic adult tissue for DAPI (nuclear marker, blue), SOX9 (ductal marker, red), GCG (alpha cell marker, green), white arrowheads showing SOX9^{cyto}-expression in cells that also express GCG. Scale bar = 20 μ m.

Discussion

Deciphering the transcriptional mechanisms underlying endocrine cell formation during human pancreatic development is essential for the optimisation of beta cell regeneration strategies. Here we show that SOX9 is expressed in the cytoplasm of a subset of human fetal beta cells, which has never been reported before, and is in contrast to the SOX9^{cyto}-expression observed in a subset of adult pancreatic alpha cells². Advanced insights have led to an increased understanding of human islet cell heterogeneity and plasticity, and it has been postulated that the SOX9^{cyto}-expression displayed in the adult pancreas might be indicative of a previously unknown islet cell plasticity². It has been observed that under certain stimuli human endocrine cells can convert their identity²¹. Furthermore, human alpha cells display a plastic epigenomic state supporting a conversion to beta cells²². However, our findings based on SOX9^{cyto}-expression in fetal tissue suggest the opposite, *i.e.*, adult alpha cells might be derived from beta cells. There have been several reports showing evidence that beta cells can change identity to alpha cells, but why this would occur in postnatal pancreata of healthy subjects is unknown²³⁻²⁶. Moreover, it is unclear what could explain the discrepancy between the different hormone positive SOX9^{cyto} cells in fetal and postnatal pancreas, it could be that plasticity potential of these endocrine cells at these stages is different, or that these are two different cells expressing SOX9^{cyto}. Lack of lineage tracing strategies for human fetal post-mortem samples prevents the collection of direct evidence for this.

In addition to the fetal endocrine cell clusters, we find SOX9^{cyto}-positive cells expressing beta cell markers in the fetal ductal epithelium, while in the adult pancreas SOX9^{cyto} is expressed in glucagon-positive cells that are located within the pancreatic duct. Could this mean that SOX9 nucleocytoplasmic shuttling is important for endocrine specification from pancreatic progenitor cells? Controlled access of proteins to the nucleus is a key driver of developmental switches and programmed cell differentiation. In mammalian gonadal development, nuclear translocation of SOX9 is the prime activator of testis differentiation, while cytoplasmic expression contributes to

ovary differentiation^{27,28}. Defects in the nucleocytoplasmic shuttling process of SOX transcription factors in humans results in the male-to-female sex reversal syndrome^{8,29,30}. In the intestinal epithelium, Wnt-signaling-stimulated upregulation of SOX9 represses differentiation genes such as CDX2 and MUC2, thereby maintaining a progenitor phenotype³¹. Human limbal epithelial stem/progenitor cells (LEPCs) have SOX9^{cyto} expression in basal LEPCs that is required for maintenance and quiescence of these stem cells, whereas nuclear translocation of SOX9 parallels proliferation and early differentiation³². It could be that loss of nuclear SOX9 prevents SOX9 from maintaining pancreatic progenitor identity and enables cells to differentiate towards an endocrine cell type. But if SOX9^{cyto} is involved in endocrine cell differentiation, it is unclear why we observe this discrepancy in fetal beta cells and adult alpha cells. It is unknown what exactly regulates the segregation of endocrine progenitor cells into the specific endocrine lineages during human development, but in-depth analysis of NEUROG3 endocrine progenitor cells in rodents show a temporal heterogeneity of endocrine progenitor cells during development³³, and lineage tracing studies show that adult beta cell heterogeneity originates from endocrine progenitor heterogeneity during development³⁴. Thus, endocrine progenitor heterogeneity might explain the discrepancy between fetal beta- and adult alpha cells, and it might be possible that a temporal stage-specific aspect (*i.e.*, fetal and postnatal) trigger differentiation of a heterogeneous endocrine progenitor population into a specific endocrine lineage.

We found no link between proliferation and expression of SOX9^{cyto} in human fetal pancreatic cells, in contrast to the previous findings in adult tissue. This could be a reflection of normal endocrine cell formation in humans, which is reported to be the result of endocrine progenitor cell differentiation during development, whereas, postnatally replication is the main contributor to new endocrine cells in humans³⁵. Other postnatal states where SOX9^{cyto} is linked to proliferation are aggressive cancers. Human pancreatic ductal adenocarcinoma (PDAC) expressing tumour protein 53 (TP53) is associated with cytoplasmic SOX9 expression in ductal cells and is clinically associated with poor disease-free survival and higher tumour grade. *In vitro* lentiviral-mediated expression of TP53^{R175H} in human PSC-differentiated exocrine organoids induced SOX9^{cyto} expression, whereas in control mCherry or KRAS^{G12V} organoids nuclear SOX9 was observed, demonstrating SOX9^{cyto} expression can be induced in specific (patho)physiological conditions³⁶. Cytoplasmic SOX9 is also a marker of higher tumour grade and poor prognosis in other malignancies, such as breast cancer and oral squamous carcinoma^{37,38}. Additionally, *in vitro* treatment of breast cancer cells with Trichostatin A, an epigenetic modifier, led to translocation of SOX9 to the nucleus, which induced growth arrest of these cells³⁹.

To our knowledge, we are the first to describe the expression of SOX9^{cyto} in the human developing pancreas. The majority of previous reports that identified the crucial role of nuclear expression of SOX9 in pancreatic multipotent progenitors and ductal cells have generated data using rodent models⁴⁰. However, it has become increasingly evident that islet biology and development, including transcriptional regulation, have interspecies differences⁴¹, which could explain why SOX9^{cyto} in the fetal pancreas has not been described before. Moreover, it could be that the current

murine antibodies do not detect the same (post-transcriptionally modified) form of SOX9 as the human antibody.

Although the mechanism of action of SOX9^{cyto} remains unclear, a strength of our study is that we were able to identify a novel SOX9^{cyto} population in human fetal pancreas development in early gestational age. It would be interesting to see from which embryonic age SOX9 shuttling occurs, and whether SOX9^{cyto} activity can be observed in earlier pancreatic progenitor cells perhaps prior to endocrine cell specification which would indicate its requirement for endocrine cell specification. In addition, it would be interesting to evaluate whether pancreata from older fetal donors also show alpha cells with cytoplasmic SOX9, similar to the early postnatal pancreas, to determine if there is a temporal aspect to SOX9^{cyto} expression in relation to endocrine cell specification. However, limitations due to current legislations, ethical review, and codes of practice limits the study of certain pancreatic development periods.

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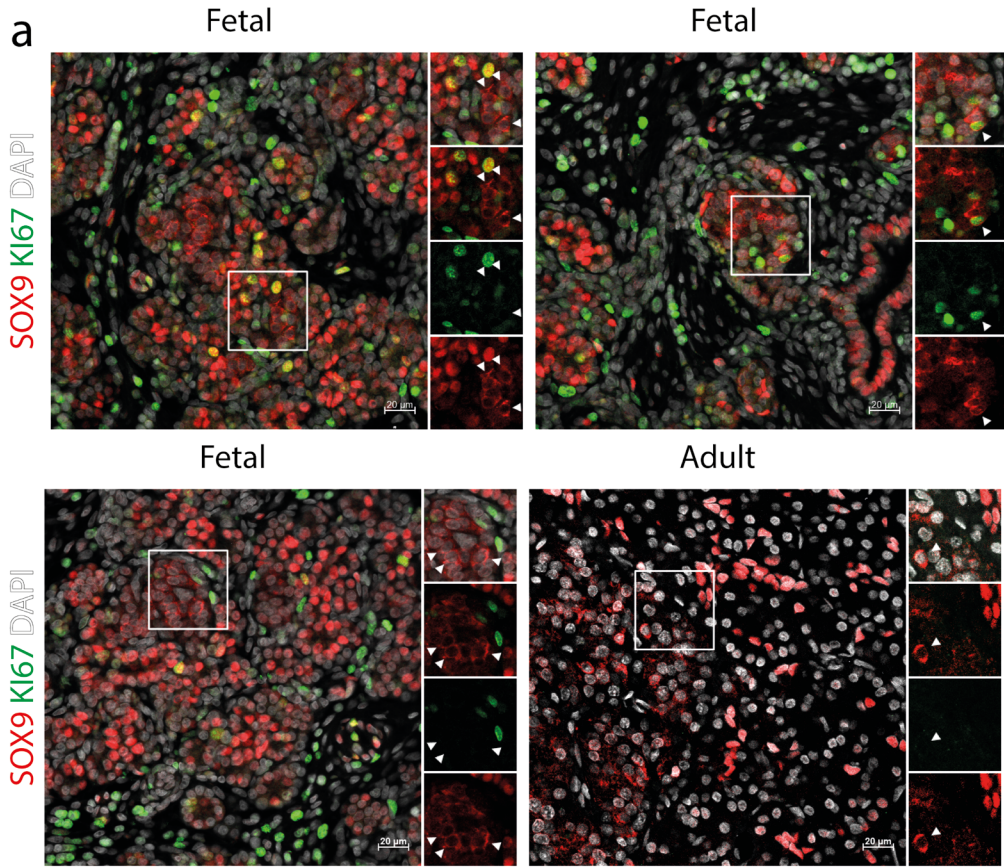
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Supplemental information

Supplemental Table 1. List of primary antibodies used

Marker	Host	Provider	Cat number	Dilution
ARX	Sheep	R&D	AF7068	1:1000
Glucagon	Mouse	Sigma	G2654	1:200
Insulin	Guinea pig	Abcam	AB7842	1:200
Synaptophysin	Mouse	Millipore	MAB5258	1:1000
Ki67	Mouse	BD Pharmingen	556003	1:200
KRT19	Rabbit	Abcam	AB52625	1:250
KRT19	Mouse	Cell Signaling	4558	1:200
NKX6.1	Mouse	DSHB	F55A12	1:1000
PDX1	Goat	R&D	BAF2419	1:10
SOX9	Rabbit	Millipore	AB5535	1:500
SOX9	Rabbit	Abcam	AB36748	1:100



Supplemental Figure 1. Ki67 is rarely expressed in SOX9^{cyto} cells in fetal and adult pancreata

(a) Immunofluorescent staining of human fetal pancreas (19 wpc) and human adult pancreas for SOX9 (ductal marker, red), Ki67 (proliferation marker, green), DAPI (nuclear marker, white), showing sporadic SOX9^{cyto}/Ki67 double-positive cells. No SOX9^{cyto} proliferating cells were observed in adult tissue. Arrowheads point to SOX9^{cyto} positive cells. Scale bar = 20 μm.