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Unraveling the Myth of Foxa2 in Endocrine Formation of the Pancreatic Lineages

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Abstract

Diabetes mellitus is a severe disease caused by autoimmune destruction and/or secreting defects of the β-cells with a global prognosis of 844 million patients in the next 20 years (The World Health Organization). The differences are stated either as T1D (Type1 Diabetes) or T2D (Type 2 Diabetes), focusing on the occurrence of the disease itself and the inheritance either early in life, at the beginning of the adulthood or in adults. Further studies are progressively made in the process of pregnancy, where a specific subset of diabetes appears for the expectant mother. Thus, making the disease itself a complex challenge in the world health population and on focus in the field of Research and Development. The focus is driven by elucidating the different factors in the maturation steps of the pancreatic insulin-secreting β-cell, impairment in this β-cells lead either to T1D or T2D. The main factor which accelerates the progression of the developing pancreas suggests being the fork head box (Fox) gene *Foxa2*, targeted deletions of Foxa2 in mice led to increased adiposity on a high-fat diet and decreased adipocyte glucose uptake and glycolysis. Interestingly, the null allele of *Foxa2* leads to severe defects in embryogenesis and death at the embryonic stage (E) 10-11, suggesting an important role in the process of organogenesis. Thereby, *Foxa2* and its target genes may shed light in specifically elucidating the transcriptional and signaling network which drives the lineage formation within the pancreas and suggests to be a promising target for creating the β-cell *in vivo*. Insights into the unique expression of *Foxa2* in pancreatic organogenesis will accelerate our understanding of pancreatic development and highlight current findings in the field of diabetes.

Keywords: Foxa2; T1D/T2D; Pancreas organogenesis; Synaptotagmin 13

Abbreviations

Akt: Phosphatidylinositol 3-kinase-Akt; BSA: Bovine Serum Albumin; Cd49f: Integrin alpha 6; CHIP: Chromatin Immunoprecipitation; Cpa1: Carboxypeptidase 1; D: Dimensional; DAPI-4',6: Diamidin-2-Phenylindol; E: Embryonic Stage; EMP: Epithelial Multipotent Progenitor; EMT: Epithelial-to-Mesenchymal Transition; FCS: Fetal Calf Serum; FOX: Forkhead Box; FVF: Foxa2-Venus; Gata: Erythroid Transcription Factor; GK: Glucokinase; GLUT2: Glucose Transporter Type 2; GWAS: Genome-Wide Association Studies; h: hours; HIGH-MAGN: Higher Magnification; HNF: Hepatic Nuclear Factor; IAPP: Islet Amyloid Polypeptide; IHC: Immunohistochemistry; INS: Insulin; min: minutes; Mist: Cytokine-Dependent Hematopoietic Cell Linker; MODY: Maturity-Onset Diabetes of the Young; NGN3: Neurogenin 3; NKX: NK Homeobox; Nkx6-1: NK Homeobox 6-1; Nr5a2: Nuclear Receptor Subfamily 5 Group A Member 2; PAX6: Paired Homeobox X 6; PBS: Phosphate Buffered Saline; PDX-1: Pancreatic and Duodenal Homeobox Factor-1; PE: Pancreatic Epithelium; PFA: Paraformaldehyde; PMP: Pancreatic-Derived Multipotent Precursors; Ptf: Pancreas Transcription Factor; RT: Room Temperature; Snail: Family Transcriptional Repressor; SNP: Single-Nucleotide Polymorphism; SOX: SRY-Related; Sox9: SRY-Related Gene 9; SST: Somatostatin; T1/2D: Type 1/2 Diabetes; TF: Transcription Factors; ZEB: Zinc Finger E-Box-Binding Homeobox

Introduction

The pancreatic primordia evaginates out of the ventral anlagen at around E9.0, sprouting into the surrounding mesenchyme. The transcriptional hierarchy of the pancreatic development is mainly described by the islet transcription factor pancreatic and duodenal homeobox factor-1 (PDX-1) [1]. The gene itself was discovered as an activator of gene expression of insulin (INS) and somatostatin (SST). In addition, PDX-1 suggests regulating GLUT2 (Glucose transporter Type 2), GK (glucokinase) and IAPP (islet amyloid polypeptide), which are identified in the β-cells of the adult Islets of Langerhans [2]. During pancreatic organogenesis, the mRNA expression of *Pdx-1* remains high until E10.5 and suggests to be downregulated in the proliferating epithelial multipotent progenitor (EMP) cells. Contrary, in the differentiating endocrine precursor cells at the embryonic stage (E) 14.5, the expression is upregulated as the cells delaminate into the EMP [3]. Crucial upstream regulators of *Pdx-1* are *Foxa1* and *Foxa2*, whereas the endocrine initiating factor *Neurogenin 3 (Ngn3)* is regulated downstream of Pdx-1 to activate the endocrine differentiation program [4-8]. Tissue-specific deletion of *Foxa2* in mice especially in the pancreas revealed a possible role of *Foxa2* in the formation of the Islets of Langerhans, respective the β-cells [9-11]. Instead of the defined architecture in the wildtype with α-cells lining the rim of the Islets of Langerhans and a core represented by ß-cells, the inner cells are randomly distributed by type and formation [12]. Interestingly, *Foxa2* itself is regulated by Insulin through Akt-mediated phosphorylation, progressively affecting the localization and thereby activation/suppression within the cell either nuclear or cytoplasmic regionalized. Also, the Akt pathway plays an essential role in insulin secretion and associated diabetic-related diseases [13]. Thus, linking *Foxa2* to T1D/T2D, in line that HNF1α, HNF1β and HNF4α are already determined by mutations as maturity-onset diabetes of the young (MODY) genes. We further postulate the relationship of mutations in *Foxa2*, which might lead to MODY; latest reports already reflected the association to reduced responsiveness to insulin with *Foxa2* as a contributor to the pathogenesis of T2D [14]. In summary, we highlight the importance of *Foxa2* in pancreatic organogenesis, especially focusing the endocrine lineage commitment. To systematically profile pancreatic lineage

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segregation, we captured live imaging pictures of single pancreatic EMP cells (*Foxa2*-Venus fusion positive) and consecutively followed temporal and spatial single cells which cluster to higher spheres-like structures. The single cells budge in *in vivo* cultures and aggregate to a higher-order consisting of EMP cells. We demonstrate, that these EMP cells inherit the pancreatic endocrine progenitors, explicitly marked by *Foxa2* upregulation, and suggest, next to established factors, new factors and mechanism in this process. Thereby, we are especially focusing on promising endocrine targets as Synaptotagmin 13 (Syt13) and highlight the co-expression to cell-cycle dependency. Thus, shedding light in pancreatic organogenesis and morphogenesis, further deciphering the importance of *Foxa2* in lineage allocation and formation of pancreatic organogenesis. Also, we hypothesize about the importance of *Foxa2* in β-cell progression as a novel gene in MODY, which may be characterized by the genetic signature itself. In summary, we expect to bring further knowledge to the pancreatic community for elucidating factors relevant to either T1D/T2D and advance current treatments in this field.

Methods

Animal handling

Mouse keeping was done at the central facilities at HMGU in accordance with the German animal welfare legislation and acknowledged guidelines of the Society of Laboratory Animals (GV-SOLAS) and of the Federation of Laboratory Animal Science Associations (FELASA). Post-mortem examination of organs was not subject to regulatory authorization.

Immunohistochemistry

The pancreas of the different embryonic stages E13.5 and E14.5 had been dissected, fixed in 4% Paraformaldehyde (PFA) for 20 min and after a subsequent sucrose gradient up to 70% sectioned into 10 µm slices on microscope slides. For IHC the pancreatic tissue had been permeabilized for 20 min (0.1 M Glycine, 0.2% Triton X-100) and incubated overnight in blocking solution containing 0.1% Tween-20, 10% Fetal Calf Serum (FCS), 0.1% Bovine Serum Abumine (BSA) and 3% donkey serum in Phosphate Buffered Saline (PBS). The first antibody was diluted in the blocking solution for 6 h at Room temperature (RT) and after 3 washing steps with PBST (0.7% Tween-20) the second antibody was incubated. Afterward washing 2x with PBST for 5 min, the tissue sections were mounted with ProLong Gold Antifade with DAPI (Invitrogen).

Primary Antibodies used: Chicken anti-GFP (Abcam # 600101215 — 1:1000), Rabbit anti-Pdx1 (NEB # 5679 — 1:300), Rat anti-Cd49f (BD # 555736 — 1:200), Rabbit-anti Sox9 (Millipore # AB5535 – 1:2000), Rabbit-anti Nkx6-1 (Acris # NBP182553 – 1:1000), Rabbit-anti Syt13 (Aviva # OAAB02896 – 1:1000), Guinea-Pig-anti Ngn3 (Gift M.Sander Laboratory – 1:1000), Rabbit-anti F-Actin (Gift R Kämmerer laboratory – 1:1000),Rabbit-anti Pax6 (Abcam # 47383 – 1:1000), Rabbit-anti Cpa1 (Biozol # 18100006 – 1:1000).

Secondary Antibodies used: goat-anti rabbit lgG 546 (Invitrogen # A11010 – 1:1000), Goat-anti Guinea-Pig lgG 633 (Invitrogen # A21105 – 1:1000), donkey-anti rat 633 (Invitrogen # A21082), Donkey-anti chicken Cy2 (Dianova # 703225155 – 1:1000).

Imaging

Images had been acquired at a Leica SP5 Confocal Microscopy, image processing accomplished by the Leica LASAF Software package. Further processing of the images was proceeded using the Image J Software package.

Live cell imaging

The pancreas of the E14.5 Foxa2-Venus mice was dissected and suspended in 0.5% BSA with Collagenase treatment. The single-cell suspension was achieved through vigorous pipetting of the suspension. Culturing of the cells was accomplished in 24-wells by VectorShield, each vial filled with 1ml culturing media consisting of 0.5% BSA and 1% Supplement. The cells were continuously live imaged in 20 min intervals under incubator condition at 37°C including 5% CO_2 at the ZeissApotom. For the Image analysis of the Brightfield and 488nm Channels, the AxioVision SEG4 Rel4.9 package was used.

Results

FVF reporter-active single cells aggregate to organoid structures

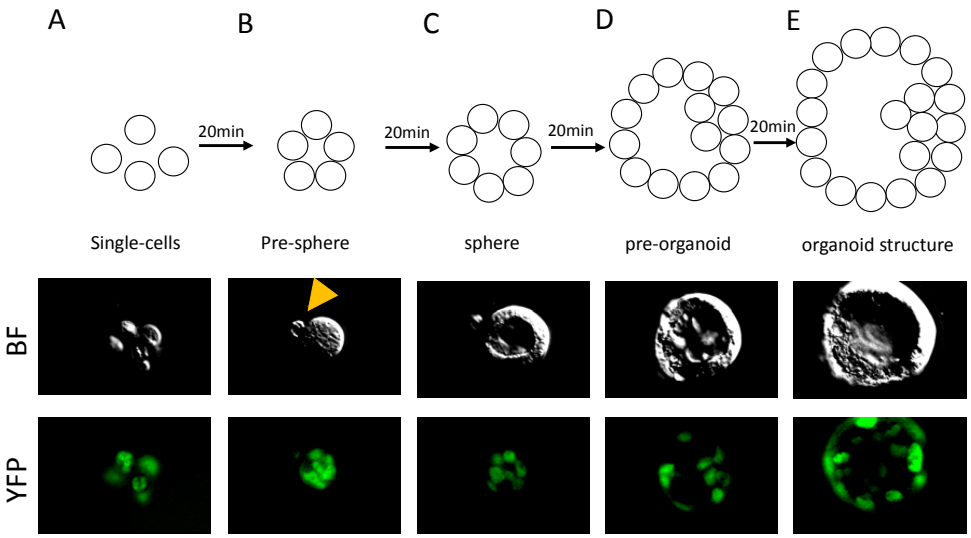
Recent evidence hints to the importance that *Foxa2* is a key regulator of mesoderm and endoderm development that has an essential role in the endoderm-specific transcription of the Pdx1 gene in the pancreatic organogenesis [15,16]. *Foxa2* is expressed at the onset of pancreatic organogenesis in the epithelial multipotent progenitor cells (EMP) and persists to adulthood in the Islets of Langerhans [17]. Thus, we utilized our recently generated Foxa2-Venus reporter mouse to investigate pancreatic organogenesis in respect to *Foxa2* [18-20]. The pancreatic epithelial marker Pdx1 illustrates co-localization to *Foxa2* in pancreatic organogenesis in the so-called secondary transition at E13.5–E15.5. Moreover, the subpopulation Foxa2-Venus^{low} in the pancreatic epithelium correlates to the Pdx1^{low} expressing epithelial cells, contrary the subpopulation Foxa2-Venus^{high} suggests to mark the endocrine progenitors (Pdx1^{high} expressing cells) in the ductal cord [18]. Under this observation, further interests are in the signals and factors which drive the EMP at E14.5 in the secondary transition into endocrine lineage segregation. Also, the potential of single cells out of the EMP pancreas to form hollow spheres-like structures at the specific E14.5 was further elucidated.

According to the last-mentioned aspect, single-cell suspension of Foxa2-Venus pancreata was accomplished by Collagenase treatment. Continuous live imaging in predetermined time intervals (20 min) revealed that single-cells cluster to a pre-spheres structure (Figures 1A and 1B). In addition, we observed EMP cells accumulated at the surface of the pre-sphere structure, suggesting the sphere structure increase by size rather due to aggregating additional EMP cells than cell division of the pre-spheres inherited EMP cells (Figure 1B, yellow arrow). Subsequent live imaging revealed spheres and pre-organoid, respective organoid structure after defined time intervals, characterized by constant Foxa2-Venus expression (Figures 1C-1E). Furthermore, it is to note, that the formerly hollow sphere (Figure 1C) form an organoid structure in a higher organization. This preliminary result might propose junctional rearrangement within the organoid structure along with polarity establishment of the EMP cells. In addition, the EMP remains Foxa2-Venus^{+} which depicts the still multipotent character of the *in vitro* organoid structure.

The onset of the EMP in the secondary transition reflects lineage segregation

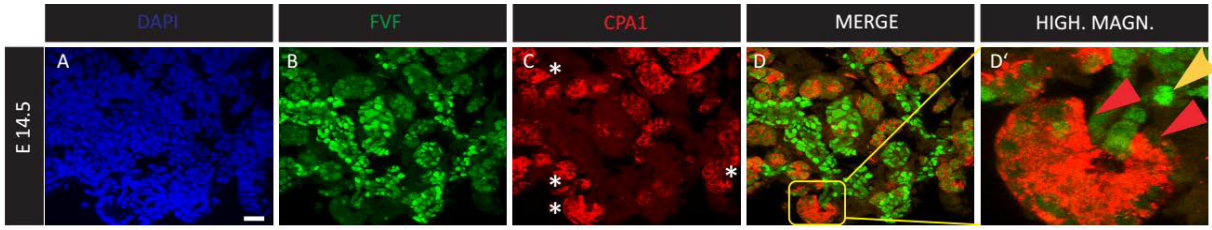
We further examined the PE of the Foxa2-Venus pancreata at E14.5 for lineage-determining factors.

The Carboxypeptidase 1 (Cpa1) has a cytoplasmic pattern in acinar cells at the ceiling of the pancreas and shows constant expression during the secondary transition. Zhou et al. already demonstrated that Cpa1



Abbreviations: nm: nanometer; E: Embryonic Stage; min: minutes; BF: Bright Field; YFP: 488 nm

Figure 1: Live imaging of E14.5 FVF single cell suspension **(A-E)**. The time-lapse imaging of the single-cells in 20 min intervals either in Brightfield (BF) or laser captured at 488 nm wavelength (YFP) represented by nuclear localization of the Venus-tag in the single cells and in the spheres like structures. The single cell suspension was achieved through Collagenase treatments of pancreata of the Foxa2-Venus embryos at E14.5 after dissection. Consecutive imaging revealed that the single cells aggregate to pre-spheres, spheres, pre-organoids and organoid structure.



Abbreviations: DAPI: 4',6-Diamidin-2-Phenylindol; FVF: Foxa2-Venus-Fusion; CPA1: Carboxypeptidase 1; HIGH. MAGN.: Higher Magnification; PE: Pancreatic Epithelium; IHC: Immunhistochemistry; E: Embryonic Stage

Figure 2: The EMP at E14.5 of Foxa2-Venus mice co-localizes to the exocrine marker Cpa1 **(A-D')**. The 4',6-Diamidin-2-phenylindol (DAPI) counterstain and immunohistochemistry (IHC) against the Venus of the Foxa2-Venus (FVF) pancreas and Cpa1 on a coronal PE section at E14.5 (A-D'). All Foxa2-Venus$^+$ expressing cells in the PE express Cpa1 at the onset of pancreas lineage segregation in the tip region (C, *). Cpa1$^+$ expressing cells are mainly in the boundary region of the PE (D). The Foxa2-Venus $^+$ compartment illustrate the subpopulations Foxa2-Venus^{high}, respective Foxa2-Venus^{low} (D', Foxa2-Venus^{high} yellow arrow, Foxa2-Venus^{low} red arrow).

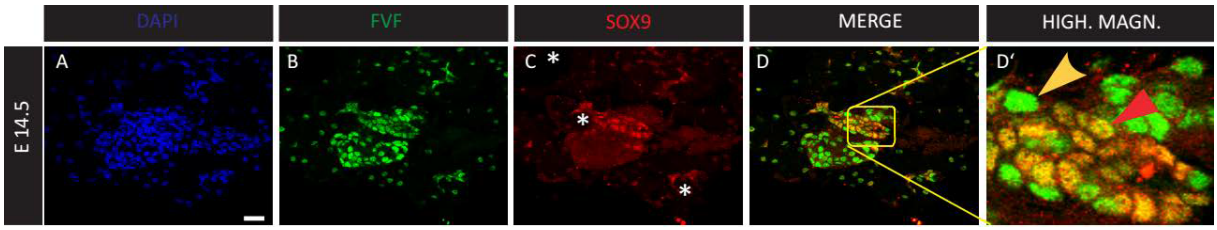
Scale is set for 25 µm

mRNA is detected at E9.5 with protein expression at E10.5 marking the EMP cells of the pancreas [21].

Interestingly, we observed expression of Foxa2-Venus in the PE along with co-expression of Cpa1 in the tip epithelial boundaries of the EMP at E14.5 (Figures 2A-2D). Also, Foxa2-Venus$^+$ cells are represented to a larger extend in the PE; all Cpa1$^+$ cells co-localize to Foxa2-Venus at E14.5 in the tip compartment of the pancreatic epithelium (PE) (Figures 2C and 2D; C *). This co-localization not only illustrates the cytoplasmic distribution of Cpa1 compared to the nuclear localization of the endogenous Foxa2-Venus protein. Also, we observed rosette-like structures representing the branching morphogenesis during pancreatic organogenesis at E14.5 (Figure 2D') [22]. It is to mention, that Cpa1$^+$ are excluded of the Foxa2-Venus^{low} compartment in the PE (Figure 2D', red arrow CPA1$^+$ Foxa2-Venus^{low}; yellow arrow CPA1$^-$ and Foxa2-Venus^{high}). In summary, our previous results do not only confirm the tip pattern of the formerly EMP in the PE. In addition, we characterized the lineage segregation into the exocrine lineage at E14.5, identified 2 subpopulations of endogenous Foxa2-Venus protein, stated as Foxa2-Venus^{high}, respective Foxa2-Venus^{low} and directly categorized a Foxa2-Venus^{low} Cpa1$^+$ cell population in the rosette-like structures as protrusions at the ceiling of the PE.

We further focused on the ductal compartment in respect to SRY-related gene 9 (Sox9). The Sox9 gene expression is excluded from the EMP cells at the beginning of the secondary transition at E12.5 and localizes in the regionalized trunk pattern [23-26]. Notably, we confirmed previous results of the Foxa2-Venus$^+$ PE, Foxa2-Venus^{low} Sox9$^+$, Foxa2-Venus^{high} Sox9$^-$ and Foxa2-Venus^{low} Sox9$^-$ cell populations in the PE at E14.5 (Figures 3A-3D, C *). The co-localization of Sox9 and Foxa2-Venus^{low} is restricted to the cord-like structure, defining the common progenitor pool of the formerly bi-potent trunk precursors (Figure 3D' red arrow). Thus, the endocrine committed cells are represented by Foxa2-Venus^{high} Sox9$^-$ expression (Figure 3D' yellow arrow), whereas the Foxa2-Venus^{low} Sox9$^+$ cell compartment reflects the ductal region.

Whereas Foxa2-Venus$^+$ expressing cells represent the PE, paired homeodomain x 6$^+$ (Pax6$^+$) cells are restricted to certain territories combined in clusters in the PE (Figures 4A-4C). More precise, the Pax6$^+$



Abbreviations: DAPI: 4',6-Diamidin-2-Phenylindol; FVF: Foxa2-Venus-Fusion; Sox9: SRY-Related Gene 9; HIGH. MAGN.: Higher Magnification; PE: Pancreatic Epithelium; IHC: Immunohistochemistry; E: Embryonic Stage

Figure 3: The EMP at E14.5 of Foxa2-Venus mice co-localizes to Sox9 **(A-D')**. In coronal sections of the PE at E14.5, the Foxa2-Venus reflects a regionalized pattern compartment marked through IHC using Sox9 (DAPI counterstain). The Sox9^{+} cell population co-localizes to the Foxa2-Venus^{+} cell population (A-D; C *). It is to note, that the FVF^{low} cells are characterized through Sox9^{+} regionally localized expressing cells. On that account, FVF^{high} cells are Sox9^{-} (D; D', Foxa2-Venus^{low} Sox9^{+} red arrow and Foxa2-Venus^{high} Sox9^{-} red arrow).

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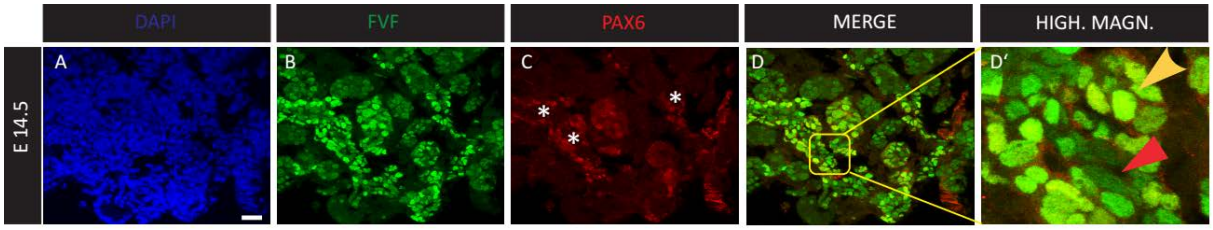


Abbreviations: DAPI: 4',6-Diamidin-2-Phenylindol; FVF: Foxa2-Venus-Fusion; Pax6: Paired Homeodomain x 6; HIGH. MAGN.: Higher Magnification; PE: Pancreatic Epithelium; IHC: Immunhistochemistry; E: Embryonic Stage

Figure 4: The EMP at E14.5 of Foxa2-Venus mice co-localizes to endocrine marker Pax6 **(A-D')**. IHC of the PE and mesenchyme (DAPI counterstain) at E14.5 on a coronal pancreatic section against Foxa2-Venus (FVF) and Pax6 (Paired homeodomain x 6) (A-D'). Pax6^{+} expressing cells are restricted to the trunk compartment in the PE (C, *). Contrary, the Foxa2-Venus^{+} compartment represents the PE. Consistently as illustrated earlier, the PE illustrates the subpopulations Foxa2-Venus^{high} and Foxa2-Venus^{low} (D', Foxa2-Venus^{high} yellow arrow; Foxa2-Venus^{low} red arrow).

Scale is set for 25 µm

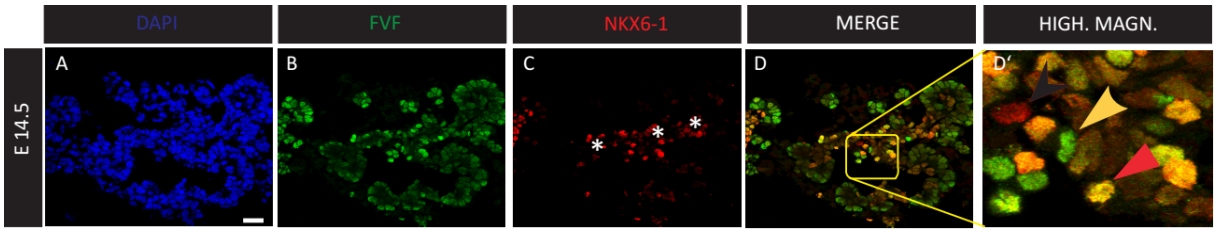
regions are within the PE, compared to the tip pattern of Foxa2-Venus^{low} Cpa1^{+} expressing compartments. Also, Foxa2-Venus^{high} co-localizes to Pax6; these regions suggest to represent the trunk pattern and thereby endocrine committed cells (Figure 4D) [27,28]. We could observe the direct correlation of Foxa2-Venus^{low} Pax6^{-} (Figure 4D' red arrow) and Foxa2-Venus^{high} Pax6^{+} (Figure 3D' yellow arrow) and thereby define the different subpopulations in the PE at E14.5 more precise.

Likewise, the endocrine progenitor Pax6, the endocrine precursor NK homeobox 6-1 (Nkx6-1) becomes restricted to the particular endocrine trunk domain in the secondary transition, especially focusing at E14.5 [28-31]. We confirmed the Foxa2-Venus^{+} expressing cell population, representing the PE and a regionalized Nkx6-1^{+} cell subpopulation illustrated by the typical trunk pattern (Figures 4A-4D; C *). The observation of the Foxa2-Venus^{high} Nkx6-1^{+}, respective Foxa2-Venus^{low} Nkx6-1^{-} verified previous results of endocrine versus exocrine lineage segregation. Remarkably, different subpopulation characterized through Foxa2-Venus^{high} Nkx6-1^{-} and Foxa2-Venus^{-}Nkx6-1^{+} (Figure 5D', Foxa2-Venus^{high} Nkx6-1^{-} yellow arrow, Foxa2-Venus^{-} Nkx6-1^{+} black arrow) suggesting temporal and spatial regulation of the transcriptional hierarchy for the generation of the subtypes of endocrine cells in line with protein expression levels of Foxa2-Venus, respective Nkx6-1.

The putative novel pancreatic marker Syt13 suggests defining the endocrine lineage

Our Foxa2 co-localization study revealed that the pancreatic lineage-determining factors are expressed within the PE in the secondary transition at E14.5. In addition, subpopulations stated as Foxa2-Venus^{low} Pdx1^{low} and Foxa2-Venus^{high} Pdx1^{high} correlate to the exocrine/ductal, respective endocrine lineage segregation in the PE [9,18,20]. Thus, we were interested in the co-localization of the putative pancreatic factor Synaptotagmin 13 (Syt13) [18]. Interestingly, we identified Syt13^{+} cells in the endocrine progenitor cell pool, co-expressed to the Foxa2-Venus^{high} Pdx1^{high} subpopulation (Figures 6A-6D', *). And indeed we could confirm previous results, a Foxa2-Venus^{high} Pdx1^{high} Syt13^{+} subpopulation in the PE at E13.5, suggesting endocrine lineage segregation (Figures 6A-6D', * and 6D, red arrow). Surprisingly, we identified a Foxa2-Venus^{high} Pdx1^{low} Syt13^{+} cell compartment, implicating a critical competence window in α- and β-cell neogenesis in the secondary transition of the pancreatic organogenesis (Figure 6D', yellow arrow).

In a next step, we further gained insights into the mechanism regarding the role of Syt13. The EMP in the PE at the beginning of the secondary transition is marked through Foxa2 and Pdx1 expression [9]. Zhou et al. [21,27] described the segregation process in the secondary transition with a tip domain characterized by Ptf1α^{+}c-myc^{+}Cpa1^{+} next to Pdx1, respective Foxa2-Venus expression. Contrary, the typical trunk domain within the PE is marked by expression of TF as Ngn3 and Nkx6-1 [21,24,25,29,32,33]. These observations implicate that within the trunk domain endocrine progenitors (Ngn3^{+}) reside next to endocrine precursors (Nkx6-1^{+}) in the process of cell delamination Therefore, we analyzed the co-expression of Ngn3 to Syt13 in the PE at E14.5 more precise to further specify the temporal and spatial control of Syt13 in regard of the endocrine lineage segregation. In a first step, we confirmed co-localization of Foxa2-Venus to Syt13, highlighting the subpopulation Foxa2-Venus^{high} directly to the Syt13^{+} cell pool (Figures 7A-7D'). In addition, different subcellular localization of Syt13 in the



Abbreviations: DAPI: 4',6-Diamidin-2-Phenylindol; FVF: Foxa2-Venus-Fusion; NKX6-1: NK Homeobox 6-1; HIGH. MAGN.: Higher Magnification; PE: Pancreatic Epithelium; IHC: Immunhistochemistry; E: Embryonic Stage

Figure 5: The EMP at E14.5 of Foxa2-Venus mice co-localizes to endocrine precursor Nkx6-1 **(A-D')**. In the PE at E14.5 on coronal sections, IHC illustrates Foxa2-Venus and Nkx6-1 (DAPI counterstain). The PE is marked with a Foxa2-Venus^{+} expressing cell compartment, Nkx6-1^{+} expressing cells are restricted to the trunk compartment of the PE (C *). The PE is characterized with the subpopulations Foxa2-Venus^{high} Nkx6-1^{-} and Foxa2-Venus^{high} Nkx6-1, (D', Foxa2-Venus^{high} Nkx6-1^{-} yellow arrow, Foxa2-Venus^{high} Nkx6-1^{+} red arrow and Foxa2-Venus^{-}Nkx6-1^{-} yellow arrow).

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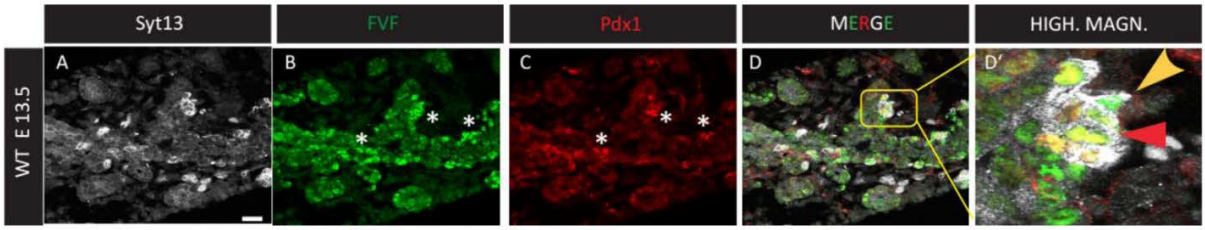

Abbreviations: FVF: Foxa2-Venus-Fusion; PDX1: Pancreatic and Duodenal Homeobox 1; E: Embryonic Stage; PE: Pancreatic Epithelium; HIGH. MAGN.: Higher Magnification; IHC: Immunhistochemistry, SYT13: Synaptotagmin 13

Figure 6: Foxa2-Venus, Pdx1 and Syt13 co-localize in the PE **(A-D').** In the secondary transition at E13.5, PE marker as Foxa2-Venus and Pdx1 illustrate the EMP (A-D'). All Foxa2-Venus^{+} expressing cells in the PE express Pdx1 at the onset of pancreas lineage segregation in the PE (C, *). A Syt13^{high} expressing compartment is mainly regionalized in the endocrine compartment of the PE and correlates to Foxa2-Venus^{high}, respective Pdx1^{high} expression (*). The endocrine lineage segregation may be characterized through Foxa2-Venus^{high} Pdx1^{low} Syt13^{+} representing the α-cells (D-D', yellow arrow), contrary the Foxa2-Venus^{low} Pdx1^{high} Syt13^{+} marks the β-cell progenitor pool (D-D', red arrow). Preliminary results suggest that the precursors of the different subpopulations α ε, δ or PP derive out of the Syt13^{+} cell pool.

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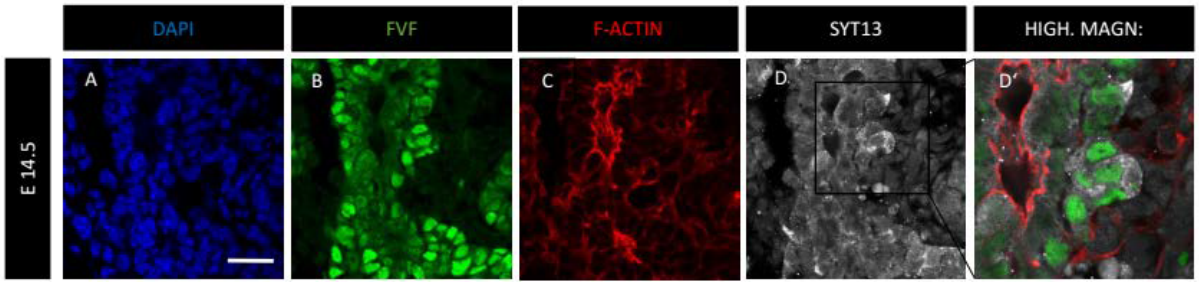


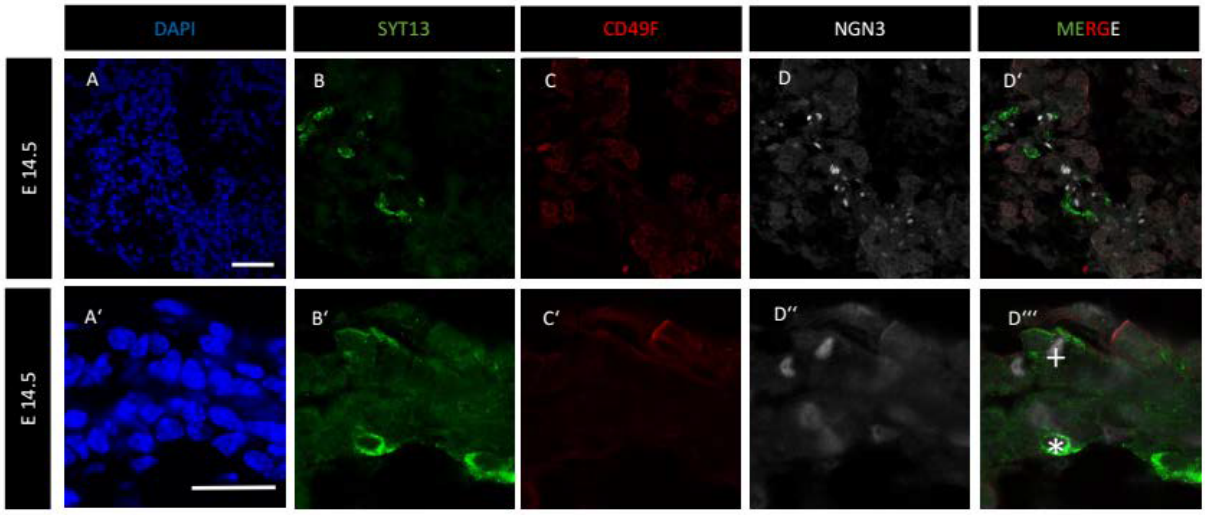
Abbreviations: DAPI: 4',6-Diamidin-2-Phenylindol; Syt13: Synaptotagmin 13; E: Embryonic Stage; PE: Pancreatic Epithelium; HIGH. MAGN.: Higher Magnification; FVF: Foxa2-Venus-Fusion

Figure 7: The Foxa2-Venus pancreata reflects Syt13 expression pattern **(A-D')**. In the secondary transition at E14.5, the PE is illustrated through DAPI counterstain, whereas F-Actin marks the ductal cord-like structure (A-DC. The Syt13^{+} cell compartments represent endocrine precursors assigned to as Foxa2-Venus^{high} (D-D"). These results indicate the involvement of Syt13 in the process of endocrine lineage segregation and further confirm localization in the endocrine progenitor pool, contrary to none in the ductal compartment.

Scale is set for 25 µm

cytoplasm of the cell was observed, either localized basal or randomly distributed within the cytoplasm of the cell (Figure 7D'). These preliminary results might suggest that Syt13 is involved in the process of delamination when the endocrine precursors leave the ductal cord.

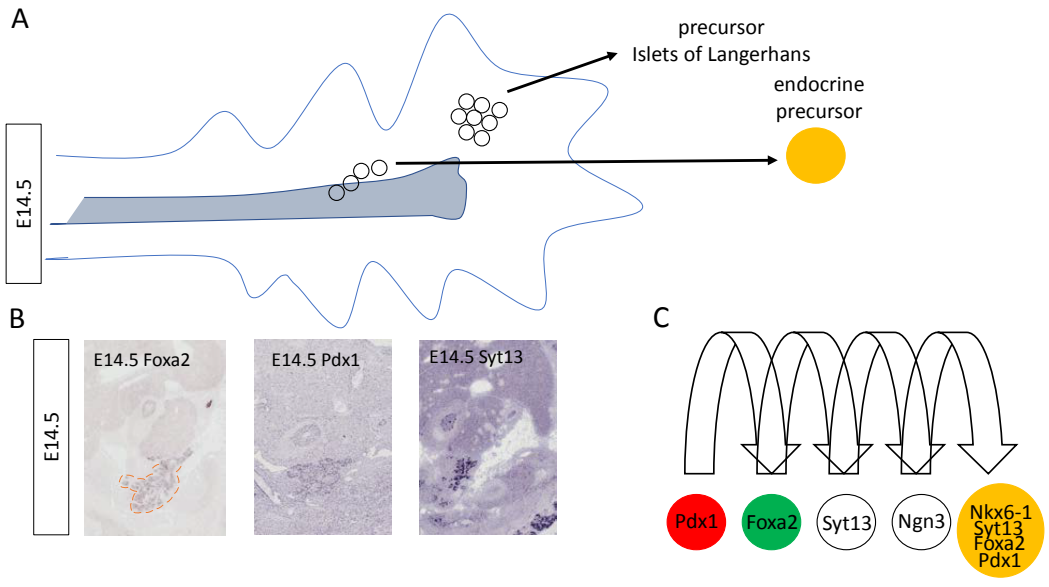
Thus, we were further interested in characterizing Syt13 in the endocrine progenitors. Previous results suggest Syt13^{+} Ngn3^{+} progenitors segregate into endocrine precursors (Nkx6-1^{+}) [12,34-41]. Previously, we identified subpopulations of Ngn3 at E14.5, Ngn3^{low} indicating the stratified ductal compartment and Ngn3^{high} indicating endocrine progenitor segregation and likely illustrating delamination of the endocrine progenitor cells out of the ductal cord (Figures 8D and 8D"). Interestingly, subcellular localization of Syt13 reflects discrepancies in the PE, in the Ngn3^{high} Syt13^{+} expressing cells basal concentration of endogenous Syt13 (Figure 8D"' +) and in endocrine precursors cytoplasmic distribution (Figure 8D"' *). Preliminary data suggests coherence of subcellular localization of Syt13 to delamination and differentiation of endocrine progenitors into endocrine precursors. We hypothesize in the process of cell division as pre-existing cellular



Abbreviations: NGN3: Neurogenin 3; SYT13: Synaptotagmin 13; CD49F: Integrin Alpha 6; E: Embryonic Stage; PE: Pancreatic Epithelium; DAPI: 4',6-Diamidin-2-Phenylindol

Figure 8: Syt13 is expressed upstream of Ngn3 positive cells **(A-D''')**. In the secondary transition at E14.5, the PE is illustrated through DAPI, whereas Cd49f marks the exocrine compartment. The Syt13^{+} cells are either Ngn3^{+} or do not express Ngn3. In addition, the Syt13^{+} cell pool either reflects random cytoplasmic distribution or basal localization. Preliminary results suggest a role of Syt13 in the process of endocrine lineage segregation, respective polarity establishment within the cell.

Scale is set for 25 µm



Abbreviations: NGN3: Neurogenin 3; FOXA2: Forkhead Box A2; SYT13: Synaptotagmin 13; Nkx6-1: NK Homeobox 6-1; E: Embryonic Stage

Figure 9: Model of endocrine formation the transcriptional hierarchy of Ngn3 and Foxa2^{high} **(A-C)**. At E 14.5 endocrine precursors leave the ductal cord and aggregate to the precursor Islets of Langerhans (A). Gene paint *in silico in situ* pattern at E14.5 illustrates a pancreatic pattern of Foxa2, Pdx1 and a cord-like pattern in the pancreatic region of Syt13. In the secondary transition at E14.5, the transcriptional hierarchy of endocrine lineage formation is mainly characterized by Pdx1^{high}, respective Foxa2^{high} progenitor cells. Upon Foxa2^{high} activation, Ngn3 characterizes the endocrine precursor cell compartment. Consequently, Syt13 is expressed in the subset of Foxa2^{high} cells and in the Foxa2^{low} expressing endocrine progenitor cells. Previous results propose a competence window in the segregation of the different endocrine precursors into α, β, ε, δ, PP cells of the immature Islets of Langerhans in line with various expression levels of Foxa2 and various Syt13 localization within the affected cell.

polarity is used to polarized cell fate determinants in a cell-autonomous fashion. During mitosis, cell fate determinants thereby segregate asymmetrically [42,43]. We have to further evaluate the impact of Syt13 in the process of delamination, respective cell division.

Based on these findings, we further postulate a model of the transcriptional hierarchy in the process of endocrine lineage formation. Thus, ductal inherited cells are triggered by factors to leave the ductal cord and aggregate in the surrounding epithelium to form the precursor Islets of Langerhans. We assume that the endocrine progenitors are marked by Pdx1^{high} *Foxa2*^{high} along with Syt13^{+} expression in the ductal cord (Figure 9A). Also, *genepaint in silico in situ* pattern showed co-expression on an mRNA level of *Foxa2* with *Pdx* in the pancreatic

region of the embryonic section at E14.5and a ductal inherited regional expression of Syt13 in the pancreatic region section (Figure 9B). Further results lead to the preliminary conclusion, that endocrine progenitor are characterized by Ngn3^{high}, in turn, endocrine precursors are characterized by Foxa2^{high} Ngn3^{low}. Upon differentiation of the progenitors into the endocrine precursor cell pool, Syt13 suggests being expressed with a various regionalized pattern in the cytoplasm within the cell. In addition, Syt13 is exclusively expressed in the adult Islets of Langerhans, implicating a role in the mechanism of endocrine maturation (data not shown). Further deciphering the exact mechanism of endocrine lineage formation, respective Syt13 might lead to the *in vivo* β-cell. We suggest a function of *Syt13* in re-establishment of the polarity complexes during the Ngn3 activated the endocrine program. Impairment in polarity establishment will lead to failure of delamination of endocrine precursors out of the ductal cord. Moreover, we propose asymmetric cell division along with partial epithelial-to-mesenchymal transition (EMT) in the process of endocrine formation. Thereby, we want to highlight the fact of Foxa2^{high} Syt13^{+} cells in pancreatic organogenesis and in embryogenesis.

Discussion

Foxa2 marks the multipotent progenitors in the pancreas

Lately published by Willmann et al., the Foxa2-Venus mouse line was utilized for a global expression profile of the pancreatic organogenesis in the secondary transition between E12.5-15.5 [18]. We confirmed the previous described Foxa2 expression in the pancreatic epithelium and identified different subpopulations in the EMP cells of the pancreas at the specific E14.5. At E14.5 lineage segregation into the different lineages of the pancreas separates the EMP into the precursor acinar and ductal cells explicitly distinguished between a Foxa2-Venus^{low}, respective Foxa2-Venus^{high} cell compartment for the endocrine compartment. We further highlight the typical tip and specific trunk pattern in the EMP for the exocrine and ductal cells, whereas the endocrine lineage correlates between this pancreatic EMP pattern [18]. Remarkably, the EMP characteristics of the Foxa2-Venus pancreatic epithelium correlates to the Pdx1 subpopulations within the EMP cells, suggesting that upstream regulator of Pdx1, Foxa2 may initiate pancreatic organogenesis [11,20]. In addition, Foxa2 is expressed in the adult Islets of Langerhans, likely in α- and β-cells (Willmann observed). Thus, emphasizing the role of Foxa2 in pancreas organogenesis and in the adult Islets of Langerhans.

Further approaches regarding Foxa2 in pancreatic organogenesis are tremendous in the aspect of lineage segregation of single cells and progenitor or even adult cell compartments, mainly meaning the Islets of Langerhans. We observed single EMP cells migrating to aggregated spheres-like structures, suggesting culturing of single sorted cells to progressively improve protocols for *in vitro* differentiation of the precursor pool into function β-cells [44-48]. To be more precise, we suggest modeling of particular pathways and factors in a temporal-spatial manner related to Foxa2 as it might decipher insulin, respective glucagon-dependent insulin secretion pathway components [49]. Taken together, the Foxa2-Venus cell population in the EMP state may represent a potential source for reprogramming of a progenitor pool into specific lineages, mainly the mature β-cells. Thus, emphasizing the role of Foxa2 as a pioneer factor in the pancreatic organogenesis [50].

Foxa2-Venus as regulator of lineage segregation in the pancreas

Pancreas lineage determinants are predominantly transcription factors (TF) which easily describe the segregation in the context of Gain/Loss of the gene of interest. On the other side, complex cell interactions via signaling and tissue interactions between the pancreas and the surrounding mesenchyme are not well understood [51-54]. To further investigate the factors affecting the lineage segregation step and signaling pathways in the different tissue compartments, Foxa2 was utilized to generate a temporal and spatial profile of the pancreas in the secondary transition at E14.5 [18]. The secondary transition is mainly characterized by complex remodeling of the epithelial sheet into a defined 3-dimensional (D) structure. Thus, metabolic pathways will establish and maintain their function in the pancreatic epithelium for a fine tuned homeostasis. The core members of the endodermal program as *hepatic nuclear factor (HNF) 1α (Foxa1), Foxa2 (HNF3β), HNF1β (Tcf2), HNF6 (Onecut1)* and *Erythroid transcription factor 4 (Gata)* are assigned to the EMP in the pancreatic organogenesis [55]. It may be speculative if *Foxa2* will be a MODY gene. Nevertheless, MODY1, MODY3, and MODY5 are determined by mutations in genes of *HNF1α*, *HNF1β* and *HNF4α*. Interestingly, *Foxa2* controls the expression of *HNF1α* and *HNF4α* and *Foxa2* itself is induced by activation of phosphatidylinositol 3-kinase-Akt through insulin [56]. As Akt/protein kinase B suggests to be involved in β-cell proliferation and insulin secretion this may point out the importance of *Foxa2* in the pancreatic β-cells [57-59]. Furthermore, *HNF1α* and *Foxa2* act upstream in regulatory domains of the *Pdx1* gene, implicating that pancreas induction is initiated through HNF1α/Foxa2 [9]. Smukler et al. [60] lately identified pancreatic-derived multipotent precursor (PMP) in human adult islets. The PMP are Insulin-positive, in line with Pdx1 and Foxa2 expressing cells. This observation may help to establish regeneration protocols for β-cell replacement therapies by selectively culturing the subpopulation of Foxa2 cells in the EMP [61-64]. In line with the previous discussion of Foxa2 as a MODY gene, might be the term T2D. In a study by Navas, T2D patients with mixed ancestry were sequenced for variations in the FOXA genes and the association to T2D susceptibility. However, no significant mutations could be detected, suggesting that FOXA members do not contribute to the disease itself [65]. Interestingly, Foxa1 and Foxa2 implicate to regulate α-cell differentiation and thereby glucagon biosynthesis and secretion in mice [66-68]. Furthermore, obviously impaired glucose homeostasis along with impaired insulin secretion in mice β- cells appears under the inducible depletion of Foxa1/Foxa2 [69]. Thus, further genome-wide association studies (GWAS) revealed variants of FOXA2 that are associated with fastening glucose levels. To be more precise, in the single-nucleotide polymorphism (SNP) at the location rs1209523, a direct acquisition of the glucose level was evident. Recently, Odom et al. combined chromatin immunoprecipitation assays (ChIP) with promoter microarrays for the identification of the transcriptional regulatory network of HNF1α, HNF4α and HNF6 in the liver and pancreatic islet tissue [70]. The results implicate that misregulation of HNF4α may contribute to late-onset T2D. In addition, FOXA2 binds to a subset of T2D susceptibility loci, underpinning the importance of the factor to in embryogenesis, respective organogenesis [71]. Taken together, our first results suggest regulation of lineage commitment and in later stages glucagon and insulin secretion by Foxa2. As Foxa2 appears to be expressed in the node and notochord common endodermal progenitors during gastrulation as Sox17 are likely to be expressed in the pancreas [20,72,73]. Although, with Sox17 suggested a role in insulin trafficking and secretion in the β-cells of normal and pathologic mouse β-cells [74]. Taken together, *Foxa2* will be a valuable factor to elucidate the pancreatic proliferation, morphogenesis and de-differentiation.

Syt13 marks the endocrine progenitor compartment

In the pancreatic organogenesis, exocrine and endocrine cells derive of a common progenitor pool, mainly cells that start to express *Pdx1/Foxa2/Ptf1α* [18,75,76]. Initiation of the pancreatic budding into the surrounding mesenchyme is composed of EMP cells which segregate in the secondary lineage to the different lineage. We identified Syt13 gene expression in the pancreatic epithelium as early as E11.5 (data not shown), suggesting that *Syt13* also characterizes the EMP progenitor pool. In the secondary transition at E13.5. The Syt13 pattern reflects a segregation into the defined trunk pattern, implicating the endocrine lineage commitment. Remarkably, the co-expression of Syt13 to Foxa2 indicates different protein expression levels – stated as $Syt13^{high}$, respective $Syt13^{low}$. Thus, the $Syt13^{high}$ cell population represents the endocrine precursors, whereas the $Syt13^{low}$ cell population represents the endocrine progenitors. Also, we may speculate about the occupancy of Foxa2 in multiple regulatory domains of the gene *Syt13*, in line with previous results for *Pdx1* [9,18]. Interestingly, *Synaptotagmin 1*, *3*, *6* and *7* are already described as a Ca^{2+} sensor to stimulate the exocytosis in the defined apical compartment of the pancreatic acinar cells [77]. Therefore, previous results could be defined more precise by characterizing the subpopulation of $Syt13^{+}$ cells in the EMP – likely the factors *Ptf1α, cytokine-dependent hematopoietic cell linker (Mist1/Bhla15)* or *Nuclear Receptor Subfamily 5 Group A Member 2 (Nr5a2)* for determining exocrine lineage allocation.

The endocrine lineage in the process of lineage segregation

The separation into the ductal/endocrine lineage leads to a trunk pattern, as illustrated for *Ngn3* [21]. The formerly EMP segregates to a lineage-specific subset of cells. In addition, basal localization of Syt13 in $Ngn3^{high}$ cells indicates a transition of polarity in the process of endocrine formation. Previous results are in line to $Ecad^{low}$ and $Syt13^{high}$ co-expression, suggesting the classical model of EMT at least partially needed for the delamination of endocrine progenitors out of the ductal cord [78,79]. Subsequent analyses of endogenous $Syt13^{high}$ implicates precursor ($Foxa2^{high}$) and mature endocrine ($Insulin^{+}$, $Glucagon^{+}$) cell commitment. In general, the loss of the epithelial program leads to the repression of E-cad, initiated by the transcriptional module of TF family *snail family transcriptional repressor (Snail)* and *Zinc finger E-box-binding homeobox (ZEB)*. In the mesenchymal state, the cells alter their front-rear polarity and start to migrate into the surrounding tissue [80,81]. Zhang et al. [82] proposed regulation of *ZEB2* by Foxa2 in breast cancer cells, highlighting the results of Burtscher et al. for the establishment of the polarity program through Foxa2 [20]. In line with this observation, upstream TF Foxa2/Pdx1/Sox9 activate transcription of *Ngn3* [83-85]. This might implicate for $Syt13^{high}$ a function in re-establishment of the polarity complexes during the Ngn3 activated the endocrine program. Impairment in polarity establishment will lead to a decrease in endocrine progenitors and failure in delamination out of the ductal cord-like structure. The functional mechanism remains unclear; we likely speculate about asymmetric cell division. Thus, we highlighted the importance of Foxa2 in the pancreatic organogenesis and suggest novel factors driving the lineage allocation within the PE, especially in focus of the im-/mature β-cells. Further characterization and concepts will shed light into the complex occurrence of either T1D/T2D.

Conclusion

The comprehensive study on Foxa2 in pancreatic development at the secondary transition as the different lineages of the pancreas segregate identified the factor *Syt13* as a factor involved in endocrine lineage commitment and thus, the importance of Foxa2 correlated signals and factors in development and maturation of functional β-cells. Preliminary results hint to a fine-tuned orchestrated hierarchy of transcription factors *Pdx1, Foxa2, Ngn3, Nkx6-1* and scaffold protein *Syt13* for the lineage allocation into endocrine in a cell-cycle dependent manner. Further deciphering the underlying mechanism will highlight current findings in the field of diabetes and eventually will give rise to the *in vitro* generated β.

Declaration

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Competing interest

The author declares that they have no competing interests.

Author contribution

SJW performed mouse work, Immunohistochemistry on pancreatic sections and analyzed the experiments. SJW wrote and drafted the manuscript. HJ designed the study.

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Ethics

Animal experimentation: Mouse keeping was done at the central facilities at HMGU in accordance with the German animal welfare legislation and acknowledged guidelines of the Society of Laboratory Animals (GV-SOLAS) and of the Federation of Laboratory Animal Science Associations (FELASA). Post-mortem examination of organs was not subject to regulatory authorization.

Consent for publication and availability of the data and material

Data will be repositioned via Dropbox and available for the public. The agreement of publication was accepted by disclosure of the Ph.D. thesis at https://www.dropbox.com/s/4ii1iqzlw65wmi9/Final%20PhDThesisSJWillmann.pdf?dl=0

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