Technische Universität München

Fakultät für Medizin

Institut für Diabetes- und Regenerationsforschung

# Understanding endoderm and endocrine lineage specification for improved stem cell-derived β-cell formation

## Katharina Friederike Scheibner

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines

## Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.
Vorsitzende: Prof. Dr. Alessandra Moretti

Prüfer der Dissertation:

1. Prof. Dr. Heiko Lickert
2. apl. Prof. Dr. Thomas Floss
3. Prof. Dr. Jovica Ninkovic

Die Dissertation wurde am 03.06.2020 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 16.02.2021 angenommen.

Technische Universität München

Faculty of Medicine

Institute of Diabetes and Regeneration

# Understanding endoderm and endocrine lineage specification for improved stem cell-derived β-cell formation

**Katharina Scheibner**

Supervisor: Prof. Dr. Heiko Lickert

**Abstract**

A comprehensive understanding of endoderm and pancreas development is the basis for *in vitro* differentiation of human pluripotent stem cells (hPSCs) into pancreatic endocrine cells. Thus, it is crucial to unravel the underlying mechanisms, such as morphogenesis and differentiation, but also signaling and transcriptional networks, that lead to the formation of the pancreatic lineages. Furthermore, human *in vitro* modeling systems are essential to study the developmental trajectory, physiology and pathology of the human pancreas for the translation into human biology and disease treatment strategies, such as cell replacement and pharmacological therapies. Therefore, in this thesis we developed an *in vitro* three-dimensional (3D) pancreatic model system and a novel approach to generate more mature and functional human stem cell-derived β-cells (SC-β-cells).

Cell replacement therapies hold great promise to cure diabetes by restoring the glycemic control in diabetes patients, however, current β-cell differentiation protocols result in heterogenous populations and immature SC-β-cells. Therefore, we established a novel approach based on the enrichment of highly specified endodermal cells to eliminate heterogeneity and to improve the yield and quality of SC-β-cells. We identified two novel surface markers, CD177/NB1 glycoprotein and inducible T cell co-stimulatory ligand CD275/ICOSL, that isolate organ progenitors specified towards pancreas and liver fate, respectively, from seemingly homogeneous endoderm differentiations *in vitro*. These organ progenitors mark subpopulations of the anterior definitive endoderm (ADE) and show differences in endoderm gene expression as well as WNT signaling. The enriched $CD177^{+}$ ADE differentiates more efficiently and homogenously into pancreatic progenitors as well as into more functionally mature SC-β-cells *in vitro*, when compared to bulk differentiations. Altogether, this study showed for the first time how fine-tuned morphogen gradients lead to patterning and fate specification of human endoderm and that enrichment of specified organ progenitors improves the generation of SC-β-cells that are more similar to their endogenous counterpart.

Studying pancreas development and its associated diseases, such as diabetes and cancer, are critical to discover novel targets for therapeutic treatments. However, such analyses are limited *in vivo*, thus human *in vitro* modeling systems are of highest importance. Therefore, we generated a simple and reproducible 3D cyst culture from pancreatic progenitors derived from embryonic mouse pancreas or from *in vitro* differentiations of human induced pluripotent stem cells (hiPSCs). The establishment of defined culture conditions enabled the survival, polarized

cyst formation, and further endocrine differentiation of mouse and human cysts. This high-resolution 3D system allowed us to monitor dynamic cellular processes such as epithelial lumen formation and endocrinogenesis *ex vivo*. Furthermore, our novel cyst system together with the analysis of published single-cell RNA sequencing (scRNA-seq) data sets revealed how the expression of transcription factors, polarity components, adherence and tight junctions change during endocrinogenesis. Altogether, this novel 3D pancreatic cyst culture offers a platform to not only study dynamic biological processes during pancreas development but also to identify underlying pathomechanisms of pancreatic diseases and new drug targets for disease treatment.

**Index**

# 1 Introduction

Diabetes mellitus is one of the most prevalent metabolic diseases that affects approximately 463 million adults with an expected increase of 629 million patients by 2045 (International Diabetes Foundation 2019). Although improved synthesis and delivery of recombinant insulin have increased survival of diabetes patients, the secondary complications and increased risk of cancer development remain a burden for patients (Fig. 1). The exponential growth of patients suffering from diabetes and its associated secondary complications urges for novel therapeutic strategies.

The two main types of diabetes mellitus are classified as type 1 (T1DM) and type 2 (T2DM). T1DM is caused by a T-cell mediated autoimmune destruction of β-cells resulting in insulin deficiency (Katsarou et al. 2017). At present, the causes inducing this disease are poorly understood, however a combination of genetic and environmental conditions are suggested. Worldwide, ~10 % of diabetes patients are affected by T1DM, which predominantly develops in children or young adults (International Diabetes Foundation 2019), while the majority of diabetes patients suffer from T2DM. The pathogenesis of T2DM is characterized by insulin resistance in insulin-target tissues resulting in β-cell dysfunction shown by impaired insulin secretion. T2DM develops due to a combination of genetic and environmental factors (DeFronzo et al. 2015). Next to the two major forms of diabetes, some patients are affected by monogenic diabetes (MD) or gestational diabetes mellitus (GDM). MD is a rare form of diabetes and identified by genetic mutations in specific genes involved in pancreatic development and/or β-cell function, such as maturity-onset diabetes of the young (MODY) (Murphy, Ellard, and Hattersley 2008). During pregnancy, an impaired response to metabolic demands leads to gestational diabetes. Several studies have shown that a rising risk of glucose intolerance and overweight occurs in the offspring of GDM mothers (Klara Feldman et al., 2016). So far, the disease mechanisms of diabetes are poorly understood, however studying pancreas development has identified new disease genes and therapeutic targets, such as MODY genes and sulfonylurea, respectively (Murphy, Ellard, and Hattersley 2008). The administration of insulin is currently the prominent therapeutic treatment for diabetes. The automated glucose monitoring and insulin delivery devices, the so-called closed-loop systems or dual hormone artificial pancreas systems, have greatly improved the insulin supply of T1DM patients (Latres et al. 2019). Although the external administration of insulin saves patient's life, they still suffer from a poor glycemic control bearing the risk of hypoglycemia, diabetic ketoacidosis and eventually lifelong secondary complications and a shortened life span. Therefore, therapeutic treatments that aim to restore the endogenous principal function of β-cells - the sensing of

glucose and secretion of insulin - are of utmost importance to cure diabetes. Cadaveric human islet transplantations reversed diabetes in T1DM patients (Shapiro 2012). However, compatible human cadaveric islets are sparse and transplanted patients require lifelong immune suppression; thus, stem cell-derived β-cells (SC-β-cells) hold great promise as an alternative for islet transplantations. The generated SC-β-cells are still poor in yield and quality; hence, it is of highest significance to understand the mechanisms coordinating the development of endoderm-derived pancreatic lineages and in particular the specification and maturation of β-cells. Furthermore, modeling the etiology of monogenic or polygenic diabetes and other pancreatic diseases will reveal underlying pathomechanisms and lead to new mechanistic understanding for novel therapies.

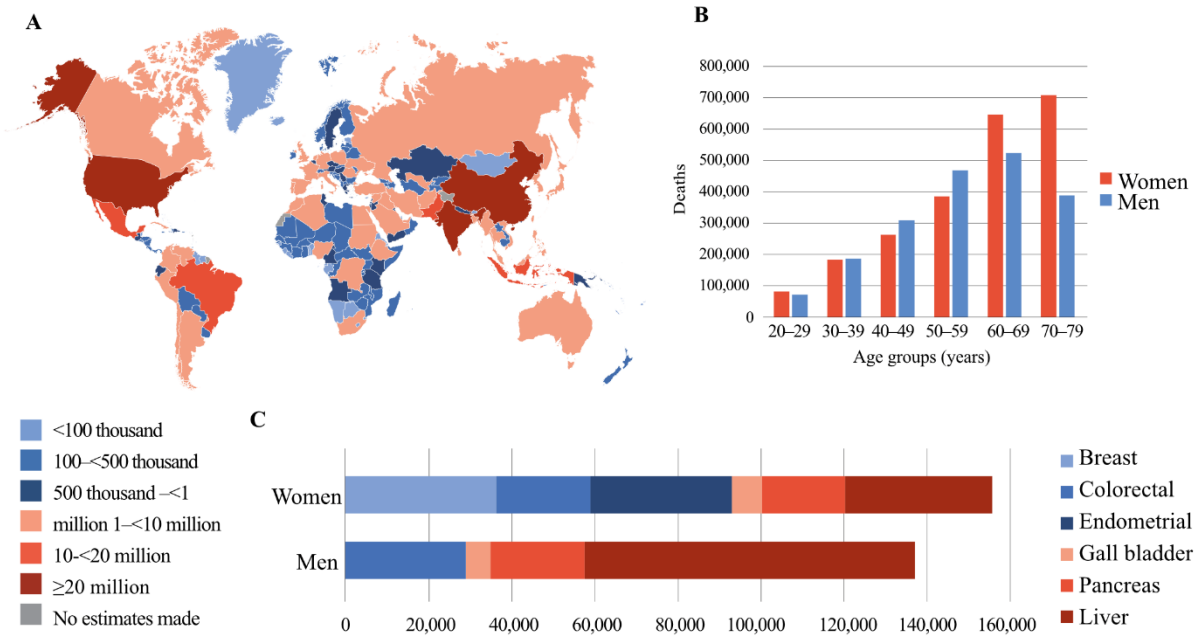


**Figure 1. Prevalence of diabetes mellitus and diabetes related mortality worldwide. A**, Estimated total number of adults (20-79 years) with diabetes worldwide in 2019. **B**, Number of death due to diabetes by age and sex in 2019. **C**, Annual numbers of cancer cases attributable to diabetes by sex. Figure modified from (International Diabetes Foundation 2019).

### 1.1. Endoderm and pancreas development

The definitive endoderm is one of the three germ layers formed during the process of gastrulation. After gastrulation, the endoderm is patterned along the anterior-posterior (A-P) axis and forms the epithelial lining of the primitive gut tube, which will give rise to endoderm-derived organ primordia such as the lung, liver, pancreatic buds and gastro-intestinal tract (Zorn and Wells 2009). The pancreatic buds develop into an organ that contains an exocrine and

endocrine compartment (Islam 2010). The exocrine part comprises ductal and acinar cells secreting digestive enzymes, while the endocrine part consists of the islets of Langerhans producing hormones that regulate glucose homeostasis.

### 1.1.1 Endoderm formation during gastrulation

The embryonic definitive endoderm (DE) derives from the pluripotent epiblast cells of the embryo during gastrulation (Nowotschin, Hadjantonakis, and Campbell 2019). Gastrulation describes the formation of the three principal germ layers, namely ectoderm, mesoderm and endoderm, via a series of cell specification, differentiation and morphogenetic events.

In the posterior epiblast, Wnt/β-catenin and TGFβ/Nodal signaling induce pluripotent epiblast cells to ingress into the primitive streak (PS) by an epithelial-to-mesenchymal transition (EMT) giving rise to the mesoderm and endoderm germ layer. It has been suggested that endoderm progenitors undergo an EMT and then re-acquire their epithelial identity by the reverse process a mesenchymal-to-epithelial transition (MET) to then intercalate into the visceral endoderm (VE), an extra-embryonic tissue surrounding the epiblast, forming the DE lineage (Arnold and Robertson 2009; Beddington and Robertson 1999; Lewis and Tam 2006; Nowotschin, Hadjantonakis, and Campbell 2019; Rivera-Pérez and Hadjantonakis 2015; P. P. L. Tam and Beddington 2007; Viotti, Nowotschin, and Hadjantonakis 2014). Endoderm formation via EMT has been shown in *Drosophila*, *C. elegans* and zebrafish, but so far has not been proven in mammals. Interestingly, a small population of endoderm progenitors was noticed in the columnar epithelium of the epiblast distal to the anterior PS (APS), that might directly intercalate into the VE without passing the PS (Fig. 2A) (Burtscher and Lickert 2009; Scheibner et al. 2019; Tam and Beddington 1992). Furthermore, live-cell imaging of differentiating reporter mouse embryonic stem cells (mESCs) that label endodermal cells showed that within ~14 hours endoderm progenitors differentiate into DE (Burtscher et al. 2012). These findings question the current view of endoderm formation, as this short time span seems insufficient for a transition from an epithelial to mesenchymal state followed by the reverse process, a MET. Indeed, we recently reported that columnar shaped epithelial DE progenitors exit the epiblast by an epithelial-to-epithelial transition (EET) and commit to a squamous shaped epithelial DE fate, independent of a classical EMT process (Scheibner et al., 2020, submitted to Nature, see attached manuscript). Furthermore, apart from mesoderm formation, EMT also causes detrimental cancer metastasis. So far, cancer metastasis was always associated with EMT, but recent studies showed an EMT-independent metastasis in pancreatic cancer, suggesting EET as an alternative mechanism of cancer metastasis. Together, this shows that understanding basic

processes of gastrulation in mammals, has broad implications for stem cell differentiation but also detrimental cancer metastasis.

The newly formed DE is patterned along its A-P axis and specified into anterior DE (ADE) and posterior DE (PDE). The endodermal sheet consists of mainly DE cells and a minor fraction of VE cells (Fig. 2A) (Nowotschin et al. 2019; Pijuan-Sala et al. 2019). However, if these extra-embryonic endodermal cells contribute to endoderm-derived organs still needs to be clarified. The entire DE expresses the transcription factors forkhead box A2 (Foxa2) and SRY HMG-box transcription factor 17 (Sox17), however Foxa2 and Sox17 are expressed in an inverse gradient along the A-P axis (Burtscher and Lickert 2009). Foxa2 expression is already detected in the epiblast before gastrulation starts and gradually increases as DE progenitors differentiate towards DE ($Foxa2^{+}/Sox17^{+}$). Foxa2 function has been shown to be crucial for endoderm formation as Foxa2 mutant embryos lack ADE, node and notochord (Ang and Rossant 1994; Weinstein et al. 1994). Ultimately, the endodermal sheet forms the lining of the primitive gut tube and is patterned along the A-P and dorso-ventral axis giving rise to fore-, mid- and hindgut spanning from the anterior to posterior body axis (Kwon, Viotti, and Hadjantonakis 2008; Nowotschin, Hadjantonakis, and Campbell 2019; Zorn and Wells 2009). The foregut forms organs such as the thymus, thyroid, lung, liver and pancreas, while the mid- and hindgut develops into the gastro-intestinal tract.

### 1.1.2 Signaling pathways specifying definitive endoderm and foregut endoderm

Prior to gastrulation, body axes are formed and morphogen gradients along the A-P axis are created. The proximal-distal and A-P body axis are induced by reciprocal signaling between extra-embryonic ectoderm, VE and epiblast secreting growth factors of the TGFβ, BMP and the FGF family (Arnold and Robertson 2009). The anterior side of the embryo is established by a global movement of VE cells to the prospective anterior side of the embryo forming the anterior VE (AVE) establishing the A-P axis at embryonic day (E) 6.0 (Beddington and Robertson 1999; Takaoka and Hamada 2011). The AVE secrets inhibitors of the TGFβ/Nodal and Wnt/β-catenin pathway including left-right determination factor 1 (Lefty1), Cerberus 1 (Cer1) and the Wnt signaling pathway inhibitor Dickkopf 1 (Dkk1) that prevent activation of TGFβ/Nodal and Wnt/β-catenin signaling in the anterior epiblast. In the posterior epiblast, signals from the Wnt/β-catenin and TGFβ/Nodal pathway induce pluripotent epiblast cells to delaminate from the epithelium and ingress into the PS giving rise to the mesoderm and endoderm germ layer (Fig. 2A).

The specification of endoderm is dependent on canonical Wnt/β-catenin signaling shown by loss of function experiments of the Wnt3a ligand, its downstream effectors and β-catenin mutants (Galceran et al. 1999; Haegel et al. 1995; Liu et al. 1999; Scheibner et al. 2019). Furthermore, the relevance of Wnt/β-catenin signaling in endoderm specification has been demonstrated by removal of β-catenin in mesendoderm progenitors in the epiblast resulting in ectopic cardiac mesoderm formation at the expense of endoderm (Lickert et al. 2002). Wnt/β-catenin signaling is not only important for endoderm induction but also for mid- and hindgut formation (Engert et al. 2013). Interestingly, foregut formation is not dependent on Wnt/β-catenin signaling indicating that a sustained Wnt/β-catenin signaling is only required for the formation of mid- and hindgut (Fig. 2B). Fate-map studies showed that already after gastrulation organ progenitors for pancreatic and hepatic lineages can be found in the foregut endoderm (Tam et al. 2006; Tremblay and Zaret 2005). It is thought that liver and pancreas progenitors derive from a multipotent ADE population and differential signaling cues from nascent tissues determines their fate (Zaret 2008). While moderate FGF signaling causes albumin expression and formation of the liver bud, low levels of FGF induce Pdx1 expression and pancreatic bud development (Serls et al. 2005). Furthermore, repression of Wnt/β-catenin pathway is required for foregut formation as well as liver and pancreas progenitor specification, as forced expression of β-catenin leads to pancreas agenesis (McLin, Rankin, and Zorn 2007; Muñoz-Bravo et al. 2016; Scheibner et al. 2019). Specification and determination of pancreatic progenitors is induced by upregulation of noncanonical Wnt signaling in foregut endoderm and pancreatic progenitors (Rodríguez-Seguel et al. 2013). Indeed, in *Xenopus* Fzd4 the receptor for Wnt5a has been shown to regulate pancreas development (Gere-Becker et al. 2018) (Fig. 2C).

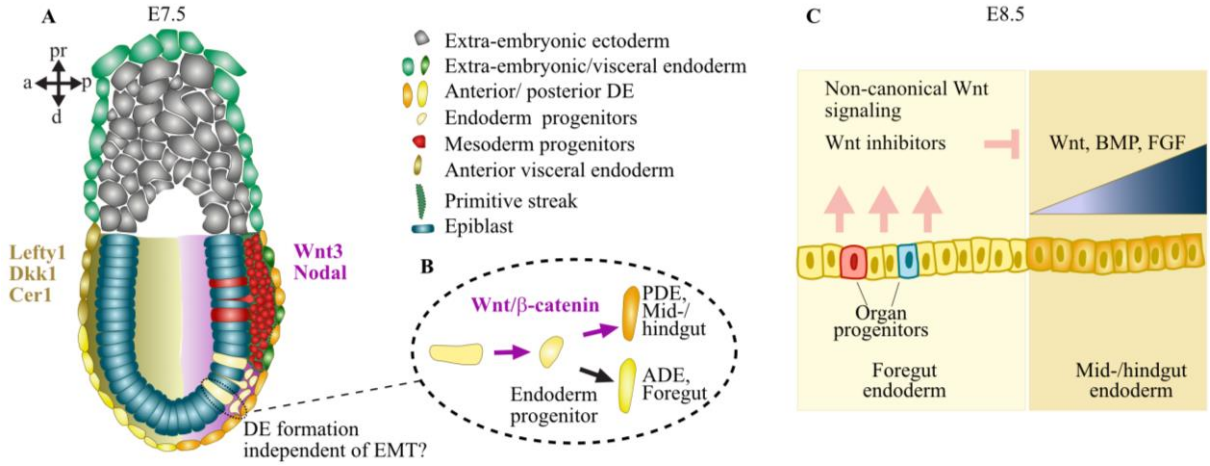


**Figure 2. Endoderm and foregut formation in mouse.** Modified with copyright permission from (Scheibner et al. 2019). **A**, Depiction of endoderm formation during mouse gastrulation. Anterior visceral endoderm cells restrict Wnt/β-catenin and TGFβ/Nodal signaling to the posterior side of the embryo, that induce the formation of the primitive streak (mesoderm progenitors). Endoderm progenitors are localized distal to the anterior primitive streak and intercalate into the VE forming a sheet of definitive endoderm divided in anterior and posterior parts. **B**, Wnt/β-catenin signaling is required for PDE and mid-, hindgut formation, while it is dispensable for ADE and foregut formation. **C**, Patterning of fore-, mid- and hindgut by (in) activation of Wnt, BMP and FGF signaling.

### 1.1.3 Endoderm specification in human

The specification of human endoderm, pancreatic organ progenitors and the signals inducing a pancreatic over liver fate remain elusive due to the unavailability at this stage of human development (Jennings et al. 2013). Human pluripotent stem cells (hPSCs) are self-renewable, can be expanded indefinitely and have multi-lineage potential, thus can generate every cell type in the human body. Therefore, hPSCs have been used to model endoderm formation and specification. Currently, the induction of DE is based on findings in mouse and thus protocols use WNT/β-catenin and TGFβ signaling pathway resulting in a seemingly homogenous DE population expressing the pan-endoderm markers CXCR4, c-Kit, FOXA2 and SOX17 (D'Amour et al. 2005; Kunisada et al. 2012). Several studies aimed to understand the fine-tuned signaling environment necessary to induce different endodermal fates. The activation of WNT/β-catenin signaling in human embryonic stem cells (hESCs) favored the expression of pan-PS markers, while a transient reduction of YAP1 and induction of TGFβ signaling induced an APS fate. Similarly, in mouse embryos YAP1 is lowly expressed in the region of endoderm formation (Hsu et al. 2018). However, TGFβ/Nodal signaling alone is insufficient for the induction of an APS or DE fate. Furthermore, a pre-exposure to WNT/β-catenin signaling is required for appropriate response to TGFβ/Nodal signaling in hESCs (Yoney et al. 2018). Comparably, the epiblast cells of mouse embryos are first exposed to Wnt/β-catenin signaling

and then followed by TGFβ/Nodal signaling resulting in the allocation towards mesoderm or endoderm germ layer (Scheibner et al. 2019; Patrick P.L. Tam and Behringer 1997). At present, the signaling niches required for directed patterning of endoderm that translates into specification of organ progenitors, important to control and optimize *in vitro* differentiation of liver and pancreatic progenitors, are unresolved for human endoderm development. However, it is questionable if such complex processes involving fine-tuned spatio-temporal patterning can be mimicked *in vitro*.

### 1.1.4 Early pancreas development

Shortly after the formation of DE and foregut endoderm, the pancreatic buds emerge at the dorsal and ventral side of the foregut endoderm. These buds consist of multipotent pancreatic progenitor cells (MPCs), that have the potential to expand and differentiate into the three lineages, acinar, ductal and endocrine and express the transcription factors (TFs) pancreatic and duodenal homeobox 1 (Pdx1) and pancreas-specific transcription factor 1a (Ptf1a) (Burlison et al. 2008; Kawaguchi et al. 2002). The absence of one of the TFs leads to pancreas agenesis in mouse (U. Ahlgren, Jonsson, and Edlund 1996; Jonsson et al. 1994; Marty-Santos and Cleaver 2015; Offield et al. 1996) and human (Stoffers et al. 1997; Weedon et al. 2013).

Pancreas development is divided in two phases, the primary and secondary transition. The primary transition starts at E8.5 and is characterized by a massive proliferation of MPCs (Burlison et al. 2008) and morphogenetic rearrangements that create a tubular network (Villasenor et al. 2010). The secondary transition occurs from E12-15.5 and is described by the segregation and differentiation of the three pancreatic lineages, acinar, ductal and endocrine.

### 1.1.5 Pancreas morphogenesis

At the beginning of the primary transition, the pancreatic buds are arranged as a multi-layered epithelium consisting of an outer layer of highly pleomorphic and motile ‘cap’ cells and mainly non-polarized stratified inner ‘body’ cells (Fig. 3A-C) (Bastidas-Ponce, Scheibner, et al. 2017; Villasenor et al. 2010). At ~E10.5 individual cells of the inner body reacquire polarity and form microlumina. The microlumina then expand and fuse to generate continuous luminal networks, that eventually form an epithelial plexus, which is remodeled into a highly branched ductal epithelium (Fig. 3D, E) (Kesavan et al. 2009; Villasenor et al. 2010). The pancreatic epithelium contains a core, in which plexus expansion (E12.5-15.5) and plexus to duct transformation (E16.5-18.5) occurs, and a peripheral region in which the epithelium is remodeled into branches. The multipotent progenitor pool is maintained by a local feedback circuit, where

neurogenin 3 (Ngn3) expressing cells repress the differentiation of neighboring cells towards an endocrine fate in a Notch-dependent manner (lateral inhibition) (Fig. 3E, F) (Magenheim et al. 2011).

The pancreatic epithelium consisting of MPCs segregates into tip (acinar) or trunk domains (bipotent endocrine/duct progenitor) starting from E11.5. The tip cells express the TFs Ptf1a and Nr5a2, while the trunk domain expresses Sox9, Pdx1, Nkx6.1 and Hnf1β (Solar et al. 2009; Zhou et al. 2007). After the endocrine cells are formed from the trunk domain, they delaminate from the epithelium, assemble in peninsulas that eventually form islets (Fig. 3F, G). The trunk domain eventually develops into mature duct cells, which express Sox9, Hes1, Hnf1β and Glis3 (Gittes 2009; Pierreux et al. 2010; Sharon, Chawla, et al. 2019).

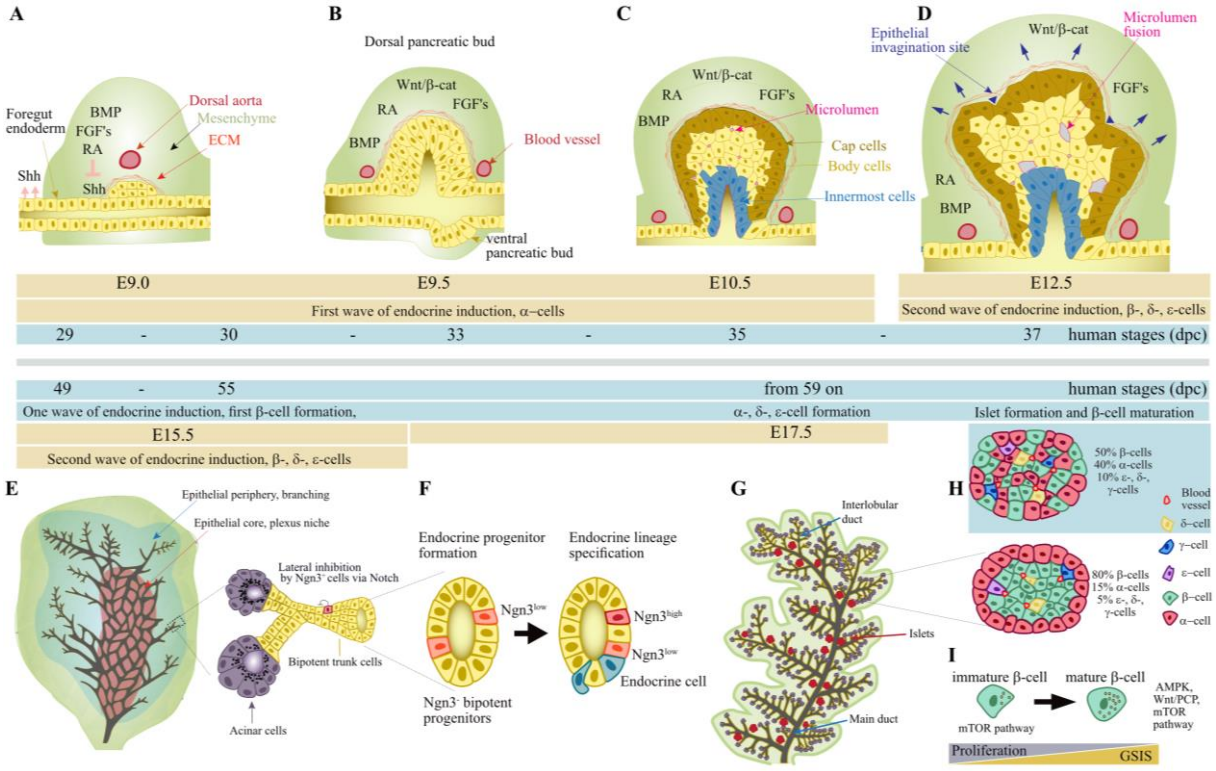


**Figure 3. Pancreas formation in mouse and human.** Modified with copyright permission from (Bastidas-Ponce, Scheibner, et al. 2017)**. A**, At E9.0 the pancreatic buds emerge from the foregut endoderm, (**B**), expand and (**C**) eventually form microlumens (**D**) that fuse and (**E**) form a tubular pancreatic network. **F**, Endocrine progenitors of the pancreatic duct differentiate into endocrine cells and (**G, H**) cluster into islets. (**I**) Different signaling pathways induce maturation of β-cells. Human development is underlined by blue boxes, while mouse development is marked by orange boxes.

### 1.2. Exocrine compartment

The exocrine compartment consisting of acinar and ductal cells composes ~95% of the pancreas and secretes digestive enzymes to the duodenum. Acinar cells derive from proacini in the distal tip of the pancreatic epithelium at ~E12.5 (Bastidas-Ponce, Scheibner, et al. 2017; Marty-Santos and Cleaver 2015). Mature acinar cells express Ptf1a, Carboxypeptidase 1 (Cpa1), amylase, elastase and trypsinogen, while mature ductal cells express Sox9, Hes1, Hnf1β and Glis3 in adult mice. Ultimately, acinar and ductal cells form a tubular network consisting of the main duct that connects to the bile duct and duodenum and further epithelial branches with acini at their tips. Disorders of the exocrine pancreas lead to diseases such as chronic pancreatitis, cystic fibrosis and pancreatic cancer (Uc and Fishman 2017). Understanding the etiology of these pancreatic diseases is limited and thus human *in vitro* modeling systems are of significant interest to analyze disease pathomechanisms and to identify novel therapeutic treatments.

### 1.3. Endocrine compartment

~95% of the pancreas consist of the exocrine compartment, while only ~5% of the pancreas belong to the endocrine compartment, namely the islets of Langerhans. In rodents, the islets of Langerhans are composed of mainly β-cells (~80%) in the inner part and α- (~15%), δ-, ε- and γ-cells (~5%) in the periphery of the islet (Islam 2010). In contrast, ~50% β-cells, ~40% α-cells and ~10% other endocrine cell types shape the human islet (Cabrera et al. 2006; Pan and Brissova 2014). In human islets, the endocrine cells are intermingled and not compartmentalized as observed in rodents (Fig. 3H).

Endocrine cells are derived from pancreatic progenitors, that generate all the pancreatic lineages (acinar, endocrine and ductal), located within the epithelial pancreatic duct. The activation and/or suppression of specific signaling pathways induces the expression of TFs important for endocrine commitment. Endocrine progenitors then allocate to an endocrine cell type by the upregulation of cell type specific TFs (Pan and Wright 2011).

#### 1.3.1. Endocrine cell differentiation

Endocrine progenitors are derived from Pdx1 and Sox9 expressing bipotent trunk epithelium. Before trunk progenitors allocate to an endocrine fate, the TF Ngn3 is transiently expressed. Ngn3 is the master regulator of endocrine cell formation as Ngn3 mutant embryos lack endocrine cells and die after birth, while ectopic expression of Ngn3 induces formation of hormone-producing cells (Gradwohl et al. 2000; Gu, Dubauskaite, and Melton 2002; Schwitzgebel et al. 2000). In the epithelial trunk, the levels of Ngn3 determine the status of the

endocrine progenitor; an endocrine biased progenitor pool ($Ngn3^{low}$) with mitotic activity giving rise to endocrine committed cells ($Ngn3^{high}$) (Bechard et al. 2016; Wang et al. 2010). The expression of Ngn3 is regulated by Notch signaling. High Notch signaling blocks the expression of Ngn3 and enhances its degradation, while low levels of Notch induce Ngn3 expression and endocrine differentiation (Apelqvist et al. 1999; Jensen et al. 2000; Lee et al. 2001; Qu et al. 2013; Shih et al. 2012). The first $Ngn3^{+}$ endocrine progenitors appear at E9, the so-called first wave, while the majority of $Ngn3^{+}$ cells arises during the secondary transition starting from E12.5 (Fig. 3). Upon endocrine commitment, Ngn3 induces the expression of endocrine-specific genes, such as NeuroD1, Insm1, Irx1,2, Rfx6, Pax4 and Nkx2.2 (Petri et al. 2006).

The endocrine progenitors become more specified towards an α- and β-cell fate by the increased expression of Arx and Pax4, respectively. Initially, endocrine progenitors express both of these TFs, however during the differentiation process the counter-inhibitory TFs are restricted to one fate eventually (Collombat et al. 2003). Further TFs such as Pax6, Rfx6, Foxa2 and MafB are essential for α-cell specification (Bramswig and Kaestner 2011). The differentiation towards β-cells requires the upregulation of the TFs Pdx1 and Nkx6.1. Both, Pdx1 and Nkx6.1 are already expressed in the pancreatic progenitors, however their expression increases and becomes restricted to mainly β-cells (Ahlgren et al. 1998; Nelson, Schaffer, and Sander 2007; Sander et al. 2000). Furthermore, the TF Nkx2.2 controls an α- versus β-cell fate, by repressing NeuroD1 in α-cells and inducing NeuroD1 in β-cells (Churchill et al. 2017; Mastracci et al. 2013; Sussel et al. 1998). Nkx2.2 expression is maintained in β-cells and prevents an α-cell program (Papizan et al. 2011).

### 1.3.2. Postnatal development, islet neogenesis and maturation of β-cells

After pancreatic progenitors differentiate into endocrine cells in the epithelial trunk domain, they delaminate from the ductal epithelium and assemble into peninsulas consisting of immature hormone positive endocrine cells (Gittes 2009; Sharon, Chawla, et al. 2019). Then the peninsulas form defined three dimensional spherical and compact islets of various sizes consisting of endocrine, neuronal, endothelial and mesenchymal cells interconnected by proteins of the extracellular matrix (ECM), cell-to-cell adhesion molecules, cell-to-matrix adhesion molecules and gap junctions. The established islet compaction, tissue architecture and polarity are crucial for endocrine cell maturation (Bader et al. 2016; Roscioni et al. 2016).

Mature and functional islet cells are essential to maintain glucose homeostasis in the body. Upon food intake mature β-cells sense glucose levels and release appropriate amounts of insulin to activate the glucose uptake in the peripheral organs such as liver, muscle and adipose tissue and thereby regulating glucose levels in the blood (Islam 2010). However, normoglycemia is assured by not only β-cells but also other endocrine cells. To prevent hypoglycemia, α-cells secrete glucagon, which mobilizes glucose from the liver. δ-cells control the secretion of insulin and glucagon in β- and α-cells, respectively (Hauge-Evans et al. 2009). Similarly, γ-cells regulate endocrine secretion but also secretion of exocrine cells (Kojima et al. 2007). The ε-cells are very sparse in adult islets, but important for satiety sensing and required for the proper control of food intake and energy expenditure (Müller et al. 2015; Tschop, Smiley, and Heiman 2000).

The immature as well as mature β-cells express TFs such as Foxa2, Nkx6.1, Pdx1, Nkx2.2, Pax4, and NeuroD1, which are necessary for β-cell formation and identity. The maturation of β-cells occurs through a postnatal maturation process. During that process, immature β-cells gain the ability to respond upon glucose stimulation in a tightly regulated manner (Asplound, Westman, and Hellersteöm 1969; Bonner-Weir, Aguayo-Mazzucato, and Weir 2016). Upon glucose stimulation, the extracellular glucose is carried into the cell through glucose transporters, the following glucose metabolism enhances the intracellular ATP levels, leading to closure of ATP-sensitive potassium channels, membrane depolarization and opening of calcium channels, and consequently increased intracellular calcium ions. Then insulin is trafficked in vesicles to the plasma membrane and the following fusion of insulin-containing vesicles with the plasma membrane results in secretion of insulin (Rorsman and Renström 2003). To acquire this functionality, β-cells need to express or upregulate a whole network of proteins, that are important for glucose sensing and insulin secretion such as TFs (MafA, Ucn3, Nkx6.1, Pdx1, NeuroD1), glucose sensors (GCK), glucose transporters (Glut2) and regulators of insulin exocytosis (Syt4, SNARE proteins, small GTP-binding Rab proteins) (Gu et al. 2010; Kaneto et al. 2009).

The TF MafB is expressed in both α- and β-cells during differentiation, however MafB expression is restricted to α-cells eventually important for their maturation and identity (Artner et al. 2007; Conrad et al. 2016). β-cell maturation requires both MafA and MafB function, however a sustained expression of MafA is essential for maturation of β-cells (Nishimura et al. 2006; Nishimura, Bonner-Weir, and Sharma 2009). Several studies showed the importance of some TFs, such as Pdx1, Foxa2, Nkx2.2 for β-cell maturation, next to their function during the

early formation of the pancreas. For instance, Foxa2, Pdx1, Nkx2.2 are involved in insulin expression and/or insulin secretion (Bastidas-Ponce et al. 2017; Brissova et al. 2002; Cissell et al. 2003; Doyle and Sussel 2007; Gao et al. 2007). Furthermore, Nkx2.2 and NeuroD1 are crucial for the expression of the glucose transporter Glut2, that allows glucose uptake and for the enzyme glucokinase (GCK) that processes glucose during glycolysis (Gu et al. 2010; Moates et al. 2003; Sussel et al. 1998). In addition, to assure a β-cell identity Nkx2.2 and Pdx1 repress an α-cell program in β-cells (Gao et al. 2014; Papizan et al. 2011). The ablation of just one of those β-cell markers disturbs β-cell identity, illustrating how critical the functional TF network is to generate and sustain mature β-cells.

Interestingly, TF activation of Nkx2.2, Pdx1 and Foxa2 is also correlated with intact islet architecture indicating that these TFs are not only important for β-cell identity but also for morphological processes in the islet (Bastidas-Ponce et al. 2017; Doyle and Sussel 2007).

### 1.3.3. Signaling pathways inducing pancreas formation and β-cell maturation

A tightly regulated signaling network coordinates the induction of the pancreas fate and its lineages. The pancreatic buds are induced by morphogens released from surrounding notochord, aortic endothelium and mesenchyme (Fig. 3A) (Wessells and Cohen 1967). Those morphogen signals include FGF2, FGF10, BMPs, retinoic acid (RA) and TGFβ/Nodal (Bhushan et al. 2001; Kim et al. 2000; Kumar et al. 2003; Martín et al. 2005; Miralles, Czernichow, and Scharfmann 1998; Norgaard, Jensen, and Jensen 2003; Wang et al. 2006; Zaret 2008). TGFβ/Nodal and FGF2 suppress sonic hedgehog (Shh) signaling to activate pancreatic gene expression (Hebrok, Kim, and Melton 1998) and RA induces Pdx1 expression independent of Shh signaling (Martín et al. 2005). FGF10 signaling is required for pancreatic progenitor proliferation and growth of the pancreatic buds by maintaining and increasing Pdx1 and Ptf1a expression (Bhushan et al. 2001). Additionally, FGF10 promotes progenitor expansion by inducing Notch signaling that inhibits endocrine specification by suppression of Ngn3 (Hart, Papadopoulou, and Edlund 2003). The expansion of pancreatic progenitors is also induced by canonical Wnt signaling (Baumgartner et al. 2014). In contrast, upon endocrine differentiation the repression of canonical Wnt signaling by expression of Wnt inhibitors and activation of noncanonical Wnt/planar cell polarity (PCP) signaling in pancreatic progenitors is needed (Cortijo et al. 2012; Larsen et al. 2015). Within the first 2 weeks after birth, β-cells mature and it has been demonstrated that Wnt/PCP signaling is crucial for β-cell maturation (Bader et al. 2016). Furthermore, the mTOR pathway has been implicated in maturation of β-

cells (Sinagoga et al. 2017). Similarly, also the AMPK pathway has recently gained attention regarding β-cell maturation (Fig. 3H, I) (Fu, Eberhard, and Screaton 2013; Salinno et al. 2019). Moreover, induction of estrogen-related receptor γ (ERRγ) expression has been suggested for the metabolic maturation of β-cells in respect to glucose-responsive insulin secretion (Yoshihara et al. 2016).

### 1.3.4. Human pancreas development

Although studying human pancreas development has been challenging due to limited human material, several studies highlighted conserved and species-specific mechanisms of mouse and human embryonic pancreas development (Jennings et al. 2013).

Prior to the formation of pancreatic buds, the dorsal pre-pancreatic endoderm is in contact with the notochord resulting in inhibition of SHH signaling in the pancreatic endoderm. During mouse development, the first Pdx1 expressing cells appear as early as E8.5 when the endoderm is still in contact with the notochord. In human, PDX1 is detected in the dorsal pancreatic endoderm after separation of notochord at 29-31 days post conception (dpc) (Jennings et al. 2013). Between 30-33 dpc the dorsal and ventral pancreatic buds emerge in the human embryo (Jennings et al. 2013, 2017; Polak et al. 2000; Slack 1995). The pancreatic buds then expand into the surrounding mesenchyme that secrets FGF7 and FGF10 inducing the growth and proliferation of pancreatic epithelial cells (Ye, Duvillié, and Scharfmann 2005). Similar to mouse pancreatic development, the human pancreatic buds consist of a stratified epithelium expressing PDX1, SOX9, NKX6.1, GATA4 and FOXA2, but the buds, and thus the pancreatic progenitors, do not express NKX2.2 (Fig. 4A) (Jennings et al. 2013; Lyttle et al. 2008). From 45 dpc onwards, the pancreatic epithelium expands and undergoes branching morphogenesis followed by ramification and formation of a lobular pattern at 49-56 dpc (Jennings et al. 2013; Polak et al. 2000). Around 52-58 dpc the two human pancreatic buds fuse and form one pancreatic anlage. Yet, the morphogenetic processes are largely unclear in human development and require further investigation.

In pancreas development of rodents two waves of endocrine induction were observed, while in human the first hormone-expressing endocrine cells are detected at 50-56 dpc (~E15.5 in mouse) suggesting that only a single wave of endocrine formation occurs during human pancreas development (Fig. 3) (Jennings et al. 2013; Lyttle et al. 2008; Riedel et al. 2012). Endocrine progenitors are born with the expression of NGN3, PAX6, NKX2.2, NKX6.1, ISLET1, NEUROD1 and PAX4 (Jennings et al. 2013; Lyttle et al. 2008; Sarkar et al. 2008). Similar to mouse development, NGN3 is only transiently expressed in endocrine progenitors

required for endocrine commitment (McGrath et al. 2015) and lengthening of G1 phase in pancreatic progenitors is crucial for NGN3 stabilization and its transcriptional activity (Krentz et al. 2017). Contrary to rodents, β-cells are the first hormone-expressing endocrine cells that arise during human development. The α- and δ-cells follow around 56 dpc and γ-cells and ε-cells at 63 dpc (Fig. 3) (Piper et al. 2004; Riedel et al. 2012).

Upon endocrine commitment, β-cells express markers such as PDX1, NKX6.1, NKX2.2, FOXA2, Insulin, NeuroD1 and PAX6 (Fig. 4A). In contrast to adult mouse islets MAFB expression is not restricted to α-cells but is expressed in both α- and β-cells in human islets. On the same lines, UCN3 is expressed in both α- and β-cells and not limited only to β-cells (van der Meulen et al. 2012). In human islets, MAFA is confined to β-cells, however the first cells expressing MAFA were noted at 4 years of age and peaked at 9 years of age different to rodent islet development. In contrast, MAFB is already expressed in juvenile islets (Cyphert et al. 2019). However, single-cell RNA sequencing (scRNA-seq) experiments of human embryonic pancreas revealed MAFA and MAFB expression already during embryonic pancreas development (Ramond et al. 2017, 2018). Thus, further research is necessary to clarify the onset of MAFA expression. The TFs SIX2 or SIX3 are not yet expressed in juvenile human islets, but they are expressed in adult β-cells and have been proposed to be involved in β-cell maturation and function (Arda et al. 2016).

To balance glucose levels in the blood, glucose sensors are essential in pancreatic β-cells. In mouse islets, the glucose transporter Glut2 is predominantly expressed and required, while in human islets GLUT1 and 3 are crucial for glucose sensing and uptake (A. et al. 2017; McCulloch et al. 2011; De Vos et al. 1995). Another protein involved in glucose sensing is the enzyme GCK important for glycolysis and following insulin release of β-cells (Matschinsky and Wilson 2019). Similar to findings in mouse, a nutrient-regulated mTOR activity is important for SC-β-cell function and maturation in human (Helman, Cangelosi et al. 2020; Sinagoga et al. 2017). The expression of maturation markers, proteins involved in glucose sensing and insulin release are hallmarks of mature and functional β-cells. Though recent years shed more light on human pancreas development many questions regarding developmental mechanisms such as progenitor specification, pancreatic morphogenesis, lineage allocation and β-cell maturation remain unanswered due to limited access to human fetal tissues and appropriate *in vitro* modeling systems.

## 1.4. Human translation: Cell replacement therapy and modeling pancreas development

A lot of progress has been accomplished to understand molecular and morphogenetic processes during pancreas development in various animal models, such as rodents and zebrafish (Bakhti, Böttcher, and Lickert 2019). However, the molecular mechanisms of human pancreas development, specifically for early developmental stages like the induction of the endoderm germ layer, formation and patterning of the gut tube, pancreas organogenesis and allocation to pancreatic lineages, is mainly unknown. *In vitro* differentiations of hPSCs to SC-β-cells and islet-like clusters (SC-ILCs) provide an attractive tool for cell replacement therapies but also to understand human pancreas development and pathogenesis of endocrine and exocrine pancreatic diseases, such as diabetes, cancer and pancreatitis.

### 1.4.1 Generation of human stem cell-derived β-cells

Human islet donors for transplantation are rare and thus generation of SC-ILCs *in vitro* from hPSCs is a promising approach to treat patients suffering from type 1 diabetes. Two decades ago, the first insulin producing cells were generated from hESCs *in vitro* (Assady et al. 2001). Since then the protocols became more defined and improved the yield and quality of SC-β-cells (Nair et al. 2019; Pagliuca et al. 2014; Rezania et al. 2014; Velazco-Cruz et al. 2019). Differentiation protocols guide hPSC by (in) activation of specific signaling pathways through different stages mimicking embryonic development, such as definitive endoderm and pancreatic progenitors to eventually fully differentiated cell types, such as β-cells. At each stage, these progenitor populations are marked by specific regulatory proteins (Fig. 4A). Although the current protocols improved significantly, the cells generated are very heterogenous and SC-β-cells are not fully mature and functional. While the first protocols were performed in monolayers, latest protocols transitioned to static or dynamic 3D approaches to better resemble the *in vivo* development. The stem cell differentiation protocols towards SC-β-cells are based on signaling pathways known to guide pancreas formation during mouse development (Fig. 4A). The first branch towards pancreatic lineage commitment, is the specification of DE arising during gastrulation by Wnt/β-catenin and TGFβ/Nodal signaling. Accordingly, *in vitro* DE formation is induced by the addition of WNT agonists in combination with the TGFβ receptor ligand activin A inducing the expression of pan-endoderm markers, such as FOXA2 and SOX17 (D'Amour et al. 2005; Kunisada et al. 2012). Following gastrulation, the DE is patterned along the A-P axis and forms the primitive gut tube with induction of the dorsal and ventral pancreatic buds coordinated by extrinsic signals from the

surrounding tissues (Zorn and Wells 2009). This process is resembled *in vitro* by activation of FGF signaling, subsequent exposure to RA and inhibition of BMP and SHH to favor a pancreatic over hepatic fate and to induce PDX1 and PTF1A expression, respectively (Hebrok et al. 2000; Martín et al. 2005; Rossi et al. 2001). To allow pancreatic progenitor expansion, EGF signaling is required as well as Notch signaling needs to be activated to prevent endocrine cell formation (Bankaitis, Bechard, and Wright 2015; Jensen et al. 2000; Löf-Öhlin et al. 2017; Rezania et al. 2014). Next, the endocrine lineage is induced and SC-β-cells formed by (in) activation of several pathways, such as the TGFβ pathway (Hogrebe et al. 2020; Löf-Öhlin et al. 2017; Rezania et al. 2014; Velazco-Cruz et al. 2019). The combination of several factors, including the antioxidants vitamin E and N-acetylcysteine (N-Cys) have been used to promote maturation of SC-β-cells (Rezania et al. 2014).

The enormous variety of these protocols including not only the differences in cytokines and durations of treatments but also different geometric and mechanical influences (2D vs 3D, static vs dynamic), results in diverse cell populations and probably heterogeneity of SC-β-cells (Fig. 4B) (Bader et al. 2016; Dorrell et al. 2016). Thus, novel approaches aim to enrich for specified progenitor or SC-β-cell populations and thereby eliminating unwanted cells and heterogeneity. Recent studies identified surface proteins marking endocrine progenitors (CD133, CD49f, SUSD2 (Ramond et al. 2017), CD200, and CD318 (Kelly et al. 2011)), pancreatic endoderm (glycoprotein 2 (GP2) (Ameri et al. 2017; Cogger et al. 2017), CD142 (Kelly et al. 2011), CD24 (Jiang et al. 2011)) and β-cells (CD49a (Veres et al. 2019), CD9, CD56, ST8SIA (Dorrell et al. 2016)) in human pancreas and/or SC-derived pancreatic cells. Isolating pancreatic GP2 expressing endoderm increased the fraction of generated mono-hormonal insulin expressing SC-β-cells (Ameri et al. 2017; Cogger et al. 2017). Furthermore, enrichment of SC-β-cells showed superior glucose response and functionality (Nair et al. 2019; Veres et al. 2019). The surface antibody screenings performed in these studies established a basis for further analysis to identify specific markers for endocrine cells. The enrichment of specified progenitor cells will reduce heterogeneity of differentiations and improve the generation of more functional SC-ILCs that resemble human islets.

Altogether, the current differentiation protocols produce heterogenous populations consisting of progenitor cells, non-pancreatic cells and endocrine cell types resulting in not fully functional SC-β-cells. A better understanding of human development by *in vitro* modeling systems would benefit for a more directed differentiation process. For instance, it is still unclear how fine-

tuned morphogen gradients specify a progenitor cell to a certain fate, like the specification of organ progenitors in the foregut or lineage allocation to α- vs β-cells.

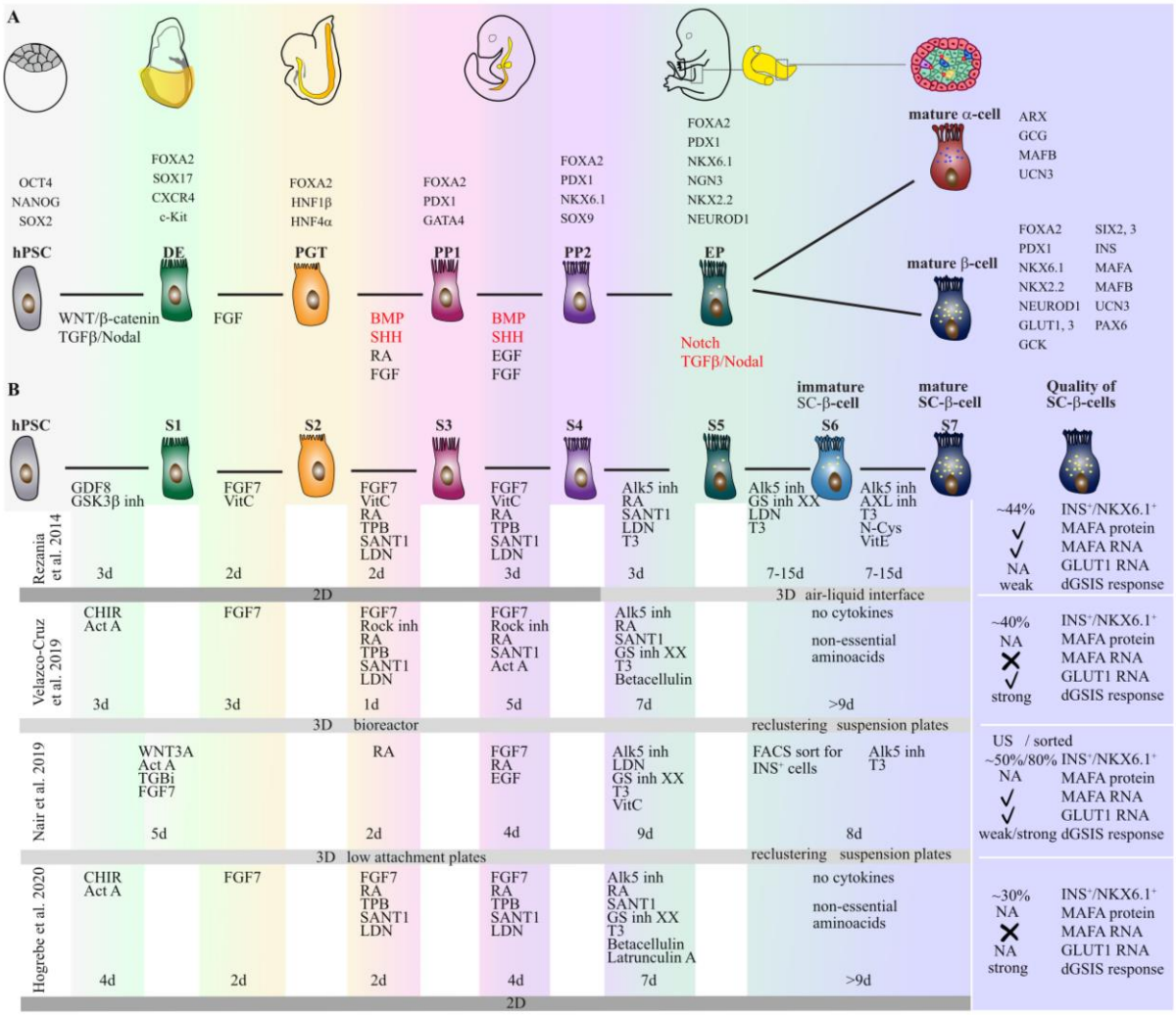


**Figure 4. Generation of human SC-β-cells. A**, Summary of β-cell differentiation protocols showing cell type-specific proteins expressed at different stages and signaling pathway activated (black) or inactivated (red) from recent publications. **B**, Assembly of prevalent differentiation protocols by (Hogrebe et al. 2020; Nair et al. 2019; Rezania et al. 2014; Velazco-Cruz et al. 2019). Figure modified with copyright permission from (Bakhti, Scheibner et al. 2019).

### 1.4.2 *In vitro* systems to model the pancreas in health and disease

The scarcity of human tissues during embryonic and postnatal stages has sparked the search for an alternative to model and understand human pancreas development and disease thoroughly. The differentiation of hPSC to pancreatic progenitors, endocrine and exocrine cell types serves as a valuable tool due to their endless availability and their human origin. Recent advances in transcriptional profiling analysis of pancreatic cells derived from *in vitro* differentiations or

fetal and adult pancreas provided a comprehensive atlas of human pancreas development. RNA sequencing of pancreatic progenitors, endocrine progenitors and endocrine cells suggested that several signaling pathways known to mediate progenitor-to-endocrine differentiation in mouse are conserved in human, including Notch (Apelqvist, 1999), hippo-Yap (Cebola et al., 2015; Gao et al., 2013; Mamidi et al., 2018; Rosado-Olivieri et al., 2019), TGFβ/Nodal and BMP (Chung et al., 2010). These signaling pathways are activated in pancreatic progenitors and are important for their expansion, but upon endocrine induction these pathways need to be inhibited. Further transcriptomic analysis discovered a novel lineage intermediate between endocrine progenitors ($NGN3^+$) and endocrine cells ($NGN3^-$) which is marked by the expression of E26 transformation-specific TF Fev (Fev), first observed in mouse (Bastidas-Ponce et al., 2019; Byrnes et al., 2018) and then confirmed in human (Krentz et al., 2018; Ramond et al., 2018; Veres et al., 2019). Though the latest single-cell transcriptomic analysis uncovered signaling pathways important for pancreatic progenitor expansion and endocrine induction, cell-ECM, cell-cell contacts and morphogenetic processes cannot be studied in such data sets. Recent work demonstrates the interdependency of morphogenesis and signaling pathways resulting in an endocrine cell fate. During endocrine differentiation, interactions between the ECM and integrin α5 of pancreatic progenitors leads to cytoskeletal remodeling that controls Yap1-Notch signaling and eventually the differentiation of pancreatic progenitors towards an endocrine fate (Mamidi et al. 2018). Similarly, changes in cytoskeleton regulates the expression of NGN3 in pancreatic progenitors; depolymerization of the actin cytoskeleton in pancreatic progenitors induces endocrine differentiation and improves β-cell differentiation (Hogrebe et al., 2020). The activation of EGFR signaling in pancreatic progenitors leads to cytoskeletal rearrangements resulting in apical narrowing, followed by downregulation of Notch and induction of an endocrine fate (Löf-Öhlin et al. 2017). Those studies highlighted the relevance of both morphogenesis and signaling pathways for endocrinogenesis.

In contrast, 3D organoid culture systems provide the natural 3D structure of tissues, and therefore critical information of cell orientation and polarity for proliferation, growth, and differentiation. Organoid cultures have been well established from several organs, such as the brain and gut (Lancaster and Knoblich 2014). In the last years, the generation of organoids from pancreatic tissues gained more attention, as they provide a platform for pancreas development, tissue transplantations, disease modeling and drug testing (Bakhti, Böttcher, and Lickert 2019). Organoids are 3D structures with functional and structural properties of the fetal or adult pancreas and derive from cells with self-renewal activity and multipotency, originated mainly from embryonic stem cells, hPSCs and organ-specific embryonic or adult progenitors (Huch

and Koo 2015). Isolated mouse embryonic pancreatic progenitors kept in a specified 3D culture condition could be expanded, branched and also differentiated into endocrine cells thus recapitulating pancreas organogenesis (Greggio et al. 2013). Interestingly, the signaling niche, like FGF and Notch signaling, was maintained in the organoid system highlighting the resemblance of the *in vitro* system to *in vivo* pancreas development. Also human fetal pancreatic cells could be expanded into duct-like structures in a 3D organoid system and differentiated towards endocrine fate in an EGF dependent manner as observed in mouse pancreatic organoids (Bonfanti et al. 2015). Furthermore, adult human pancreatic tissue was cultured and expanded in a 3D system and disclosed a subpopulation of pancreatic progenitors with higher similarity to fetal progenitors than adult pancreas. This progenitor subpopulation differentiated into the endocrine lineage upon stimulation *in vitro* and upon transplantation *in vivo*, indicating reprogramming of a subset of adult pancreatic cells to a progenitor-like stage during *in vitro* expansion. Similarly, in adult mouse islets a protein C receptor positive ($Procr^{+}$) expressing progenitor population has been identified that has the potential to differentiate into all endocrine cell types *in vitro* (Wang et al. 2020). However, if such a $Procr^{+}$ progenitor population exists in adult human islets still needs to be addressed. As human tissues are sparse, a recent study used hESCs to create pancreatic organoids resembling acinar and ductal progeny (Hohwieler et al. 2017). Remarkably, this hESC-derived pancreatic organoid system has been used as a model for cystic fibrosis *ex vivo* and could be used as a platform for drug screening.

During endocrinogenesis, cytoskeletal rearrangements in pancreatic progenitors lead to their differentiation towards an endocrine cell and delamination from the pancreatic duct (Hogrebe et al. 2020; Löf-Öhlin et al. 2017; Mamidi et al. 2018). The current pancreatic organoids with their complex epithelial structures impede the analysis of dynamic changes in cell polarity, adhesion and lineage allocation in a spatio-temporal manner. Accordingly, smaller and less complex epithelial structures so-called cysts or spheres, consisting of polarized epithelial cells forming a ball-like structure with a central lumen, are promising tools to investigate such processes at high-resolution (Bonfanti et al. 2015; Sugiyama et al. 2013). Still, endocrinogenesis has not been analyzed comprehensively. Therefore, modeling of the human pancreas holds great promise to uncover signals of the endogenous niche important for endocrinogenesis and thus could advance current β- and α-cell differentiation protocols.

## 2 Aims of the thesis

Understanding human pancreas development from endoderm formation to endocrine lineage formation and maturation will advance the generation of functional β-cells for cell replacement therapy, will help to understand disease initiation and progression and will uncover novel drug targets to treat patients suffering from diabetes.

Therefore, in this thesis, I addressed the following aims:

**Aim 1: Generation of pancreatic β-cells from CD177$^{+}$ anterior definitive endoderm.**

Currently, it is impossible to identify and isolate highly specified endodermal subpopulations that are specific for pancreas and liver fate for directed differentiation of hPSCs to pancreatic and hepatic lineages *in vitro*. Therefore, we aimed to dissect endoderm heterogeneity and to explore the potency of endoderm subpopulations. Furthermore, we wanted to understand the signaling requirements for the induction of a pancreatic fate. Overall, we wanted to establish a protocol that is more robust and improves the generation of pancreatic progenitors and SC-β-cells with superior functionality.

**Aim 2: Establishment of a high-resolution 3D modeling system for studying pancreatic epithelial cell biology *in vitro*.**

Studying the development of the endocrine and exocrine pancreas but also its associated diseases, such as diabetes, cancer and fibrosis, is critical to discover novel targets for therapeutic treatments. The detailed analysis of such developmental processes and drug screenings are limited in mouse and impossible in humans *in vivo* and restricted with the current *in vitro* modeling system. Therefore, we aimed to establish a mouse and human 3D pancreatic modeling system for the translation and/or comparison of principal mechanisms in mouse and human pancreas development. We wanted to generate a modeling system that is easy, reproducible and mimics the human and mouse pancreatic epithelium as defined epithelial cyst cultures. Furthermore, the system should allow to monitor and unravel dynamic processes that are crucial during pancreas development and disease progression in a high resolution and time-resolved fashion on a cellular and subcellular level.

## 3 Methods

**Animal studies**. Mice were kept and experiments performed at the central facilities at Helmholtz Zentrum München German Research Center of Environmental Health in compliance 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).

### Cell sources

Human islets were obtained from the Rudbecklaboratoriet C11 (Uppsala, Sweden) and islet core facility (Edmonton, Canada). The Mel1-NKX6.1-GFP were received from Australian Stem Cell Centre (Clayton, Victoria). The H1 and H9 hESC lines were obtained from WiCell Research Institute, Inc. (Madison, WI). An episomal reprogrammed HMGUi-001 iPSC line was generated from control group of MODY-4 patients (Wang et al. 2016) (Gibco Human Episomal iPSC). All cell lines have been authenticated by Csell Line Genetics (Madison, WI) and confirmed to be mycoplasma-free. The hESC lines were used with the permission of Robert Koch Institute. For mESCs experiments $T^{GFP/+}$; $Foxa2^{tagRFP/+}$ mESCs, FVF mESCs (Burtscher et al. 2013), IDG3.2 mESCs (Hitz et al. 2007), Snail1 KO mESCs, Foxa2 H2B Venus mESCs (Cernilogar et al. 2019) were used.

### Embryology

The uterus from pregnant mice was cut and transferred into DPBS. With dissecting tools the embryos (E6.0-8.5) were separated from the uterus and the decidua was opened with forceps. The Reichert's membrane had to be removed to allow the access of antibodies to the embryos. The embryos were transferred with a pipette to a glass flask containing fixative. The IHC of the embryos was processed as described below.

### Differentiation of hPSC towards β-like cells

hESCs and HMGUi-001 iPSCs were cultured on 1:30 diluted Geltrex (Invitogen) in StemMACS iPS-Brew medium (Miltenyi Biotec). At ~70% confluency, cultures were passaged by EDTA (Applichem) treatment every 3-4 days. For differentiations, cells were seeded at ~$1.5–2\times10^5$ cells/$cm^2$ on Geltrex-coated surfaces. Cultures were fed every day with iPS-Brew medium and differentiation was initiated 24 hours following seeding, resulting in ~90% starting confluency. The cells were differentiated towards definitive endoderm using MCDB131 medium (Gibco) supplemented with 0.5% BSA (Sigma), 100 ng/ml Activin A and 25 ng/ml

WNT3A (Peprotech) or 3 µM CHIR-99021 (Miltenyi Biotec) for the first day. For the next 2 days, cells were treated with MCDB131 supplemented with 0.5% BSA and 100 ng/ml Activin A.

For differentiation towards β cells, a published β-cell differentiation protocol was used (Rezania et al. 2014). Briefly, the cells were differentiated towards primitive gut tube with MCDB131 supplemented with 0.5% BSA, 50 ng/ml of FGF7 (Peprotech), 0.25 mM ascorbic acid (Sigma) and 1.25 µM IWP2 (Tocris) for 2 days. For the WNT signaling activation experiments only, 20 ng/ml of WNT3A, 3 µM CHIR, 1.25 µM IWP2 or 100 ng/ml WNT5a was added to the cultures along with the S2 medium without IWP2. For differentiation towards posterior foregut, the cells were further exposed to MCDB131 medium supplemented with 1X Glutamax (Gibco), 2% BSA, 0.25 mM ascorbic acid, 50 ng/ml FGF7, 0.25 µM SANT-1, 1 µM retinoic acid, 100 nM LDN193189, 1:200 ITS-X and 200 nM TPB for 2 days. The cells were then further differentiated using MCDB131 supplemented with 1X Glutamax, 10 mM final glucose concentration, 2% BSA, 0.25 mM ascorbic acid, 2 ng/ml FGF7, 0.25 µM SANT-1 (Sigma), 0.1 µM retinoic acid (Sigma), 200 nM LDN193189 (Sigma), 1:200 ITS-X (Gibco) and 100 nM TPB (Merk) for 3 days. For induction of pancreatic endocrine precursors, the cells were exposed to MCDB131 medium supplemented with 1X Glutamax, 20 mM final glucose concentration, 2% BSA, 0.25 µM SANT-1, 0.05 µM retinoic acid, 100 nM LDN193189, 1:200 ITS-X, 1 µM T3 (Sigma), 10 µM ALK5 Inhibitor II (Enzo life sciences), 10 µM zinc sulphate (Sigma) and 10 µg/ml heparin (Sigma) for 3 days. Hormone positive cells were generated by exposing the endocrine progenitors from the last step with MCDB131 supplemented with 1X Glutamax, 20 mM final glucose concentration, 2% BSA, 100 nM LDN193189, 1:200 ITS-X, 1 µM T3, 10 mM ALK5 Inhibitor II, 10 µM zinc sulphate and 100 nM gamma secretase inhibitor XX (Merck) for the first 7 days. For maturation of β-like cells, the cells from previous stage were treated with 2% BSA, 1:200 ITS-X, 1 µM T3, 10 µM ALK5 inhibitor II, 10 µM zinc sulphate, 1 mM N-acetylcysteine (Sigma), 10 µM Trolox (EMD), 2 µM R428 (SelleckChem) and 10 mg/ml of heparin for 15 days.

**3D culture of stem cell-derived human pancreatic progenitors**

To generate cysts, 8-well ibidi chambers (Ibidi) were coated with Matrigel (5 µl/well) (BD Biosciences) and kept for 15 min at 37°C to polymerize the Matrigel. Human differentiated iPSCs at different stages (DE, PGT, PP1, PP2 and EP) were trypsinized with 1:1 0.05% Trypsin or EDTA and DPBS (Invitrogen) for 3 min at 37°C. Subsequently the enzymatic reaction was neutralized, and cells were seeded in differentiation medium containing 10 mM Y-27632 and

5% Matrigel. The next day, the medium was replaced with the appropriate differentiation medium.

**MACS sorting of ADE subpopulations**

On day 3 or 4 of differentiation, cells were collected and stained for surface markers CD177 (BD Biosciences, Miltenyi Biotec), CXCR4 (Miltenyi Biotec) and CD275 (Miltenyi Biotec). For staining of the surface markers, 10 µl antibody was added per 1 x $10^6$ cells in 100 µl volume of MCDB1 + 0.5% BSA. The cells were stained in dark for 15 min on ice. Next, the stained cells were washed 1 x with PBS to remove the antibody and then suspended in 80 µl of MCDB131 + 0.5% BSA medium with 20 µL of Anti-PE/APC Microbeads (Miltenyi Biotec) per 10 x $10^6$ of total cells. The cells were incubated for 15 min at 4°C. The cells were washed with PBS and then suspended up to 20 x $10^6$ cells in 500 µL of MCDB131+0.5% BSA and proceeded with magnetic sorting. After MACS, the cells were seeded in iPS-Brew medium supplemented with 10 µM Y-compound at the seeding density of 2-10 x $10^3$ cells in 1 well of ultra-low attachment round bottom 96 well plates to form an aggregate or 4 x $10^5$ cells in one well of ibidi chamber for further differentiation and staining.

**Cell culture and differentiation of mESCs**

Mouse ESCs were cultured on mitomycin c-treated mouse embryonic fibroblasts (feeders) in DMEM (Life Technologies) supplemented with 15 % FCS (PAN Biotech) , 0.1 mM β-mercaptoethanol (Life Technologies), 2 mM L-glutamine (Life Technologies), 1X non-essential amino acid (Sigma), 2 mM HEPES (Life Technologies), and 1000 U/ml leukemia inhibitory factor (LIF; Sigma). Every 2-3 days cells were passaged by detachment of cells with 0,05% Trypsin (Life Technologies) on new feeders and the medium was changed every day. For differentiations towards endoderm, the Snail1 KO mESCs, $T^{GFP/+}$; $Foxa2^{tagRFP/+}$ mESCs, IDG3.2 mESCs 1 x $10^5$ cells/ 1 $cm^2$ were plated in chemically defined medium as published recently (Mfopou et al. 2014). Endoderm was induced 24 hours after seeding by addition of 2.5 µM CHIR99021 (Miltenyi Biotec) for 1 day and 25 ng/ml Activin A (Peprotech) for all 3 days. For the Wnt inhibitor experiments, endoderm was induced as described before and on day 2 and 3 400 ng/ml DKK1 (Peprotech) or 1.25 µM IWP2 (Tocris) were added. Differentiations of the $Foxa2^{Venus/Venus}$ KO mESCs were performed as described before (Cernilogar et al. 2019).

**FACS sorting of differentiated mESCs and embryonic pancreas cells**

Differentiated Foxa2$^{Venus/Venus}$ mESCs were trypsinized by 0,05% EDTA for 5 min and single cells stained with DAPI for dead cell exclusion and FACS sorted for GFP$^{+}$ cells based on undifferentiated cells.

The pancreas of embryonic Ngn3-Venus fusion (NVF) reporter mice were kept in 0.25% Trypsin for 5 min on ice and then incubated at 37°C for 10 min to generate a single cell solution. The single cell solution was then centrifuged at 1500 rpm for 5 min at 4°C. Then, 5 µl anti-mouse CD326 (EpCAM) PE (eBioscience, 12-5791-81) and rat IgG2a K isotype control (eBioscience, 12-4321-42) were used for 1 × $10^6$ cells in 100 µl total volume. The cells were stained for 30 min at 4°C, followed by a DAPI staining to detect dead cells. The cells were washed twice and resuspended in FACS buffer (PBS, 1% BSA, 0.5 mM EDTA) and loaded for FACS sorting. GFP$^{+}$ and EpCAM$^{+}$ cells were sorted based on unstained samples and isotype controls.

**Sequential static glucose stimulated insulin secretion**

Sequential static glucose stimulated insulin secretion (seqGSIS) of the generated β-like clusters was performed based on a previously described protocol (Rezania et al. 2014). 5 aggregates were picked and rinsed three times with KRBH buffer (129 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_2$, 1 mM $Na_2HPO_4$, 1.2 mM $KH_2PO_4$, 5 mM $NaHCO_3$, 10 mM HEPES and 0.1% BSA in deionized water and sterile filtered and then equilibrated in KRBH buffer at 37°C for 30 min. Then aggregates were incubated in KRBH buffer supplemented with 2.8 mM glucose for 30 min at 37°C. Supernatants were collected and the aggregates were transferred to KRBH buffer supplemented with 20 mM glucose for 30 min. Supernatants were collected again. The aggregates were then washed to remove left over high glucose with KRBH and another round of low glucose and high glucose stimulus was performed. To normalize the seqGSIS, cell aggregates were dissociated into single cells and the cell numbers were counted. Insulin content was measured by human Insulin ELISA kit (Mercodia) following the manufacturer's protocol.

**Dynamic glucose stimulated insulin secretion**

25 SC-derived islet-like clusters (30,000-50,000 cells) or human islets from healthy donors were pre-incubated in KRBH buffer containing 2.8 mM Glucose for 30 min and then loaded on a nylon filter in a plastic perfusion chamber containing acrylamide-based microbead slurry (Bio-Rad Laboratories). The SC-derived β-cells or human islets were then sequentially

perifused with low glucose (2.8 mM) for 12 min, followed by high-glucose (20 mM) for 24 min, Exendin-4 (10 nM) + high glucose (20 mM) for 24 min, low glucose (2.8 mM) for 12 min and a final step with 25 mM KCl for 12 min at a constant flow rate of 100 µl/180 sec using the BioRep perifusion system maintained at 37°C in a temperature controlled chamber. Flow through fractions were collected on a 96-well plate maintained at 4°C and quantified for insulin content. For normalization, SC-derived β-cells or human islets were recovered from perifusion chambers and assayed for DNA contents and quantified using Quant-IT PicoGreen dsDNA kit (Thermo Fischer).

**Insulin content**

S7 clusters from CD177 and unsorted differentiations were washed with PBS and dissociated using Accutase. Cells were counted and 1000 cells were collected for measuring insulin content. The cells were resuspended in Acid-EtOH solution (1.5% HCL and 70% EtOH) and kept on a shaker at 4°C overnight. The tubes were centrifuged at 2100 g for 15 min and supernatant was collected and neutralized with an equal volume of 1 M Tris (pH 7.5). Human insulin was measured as mentioned above.

**Immunofluorescence stainings of cells and human pancreatic epithelial cysts (hPECs)**

Differentiated cells were washed with PBS and fixed for 15 minutes with 4% PFA. Subsequently, cells were permeabilized by 0.1 M glycine and 0.1% triton X-100 in MilliQ water for 15 minutes at RT and blocked for 1 hour in blocking solution containing 0.1% Tween-20, 10% heat inactivated FCS, 0.1% BSA and 3% donkey serum at RT on a shaker. Primary antibodies were diluted in blocking solution and kept overnight at 4°C and another hour the following day at RT on a shaker. Cells were washed 3 x with PBS for 10 min each and subsequently secondary antibody solution was added for 2-4 hours. DAPI/PBS solution was added for 10 minutes and afterwards cells were washed 3 x with PBS for 10 minutes each. Cells were kept in PBS for immediate imaging.

**Immunofluorescence stainings of whole mount embryos**

E6.0-8.5 fixed embryos were rinsed 2 x with DPBS containing 0.1 % Tween 20 (Merck) (DPBST). The embryos were permeabilized for 10 min (≤E7.5) to 15 min (>E7.5) as described above. The permeabilized embryos were incubated for ~1 hour in blocking solution before primary antibodies were added. Subsequently, embryos were kept at 4 °C overnight on a shaker and another 1-2 hrs at RT the following day. The embryos were rinsed 2 x and washed 3 x 10

min with DPBST. The secondary antibodies diluted in blocking solution were added to the embryos for ~3 hours at RT on a shaker. The secondary antibodies were replaced by a DAPI/DPBST solution and incubated for 20 min at RT. The embryos were rinsed twice and 3 x 10 min washed with DPBST before dehydrated in 15 % and 30 % glycerol in DPBS, each 10 min at RT. Afterwards, embryos were embedded in antifade between two cover slips by using a 100 µm spacer, dried at RT and were stored at 4° C until imaging.

**Immunofluorescence stainings of cells for FACS**

Differentiated cells were dissociated using Accutase or 0.05% Trypsin (Sigma) and fixed in 4% PFA for 10 minutes. The cells were permeabilized for 15 minutes and blocked for 1 hour at RT (see above). Next, primary antibodies were diluted in blocking solution and incubated for 3-4 hours at RT or overnight at 4°C. Cells were washed 3 x with PBS for 10 min each and secondary antibody solution was added for 1-2 hours at RT. After another washing of 3 x with PBS, samples were analyzed by BD FACS Aria III. The gates were determined by secondary antibody controls.

**Cryosections of differentiated islet like clusters**

S6 or S7 clusters were fixed with 4% PFA for 15 min at RT on a shaker. For cryoprotection of the samples, clusters were incubated in 10% and 30% sucrose in PBS for 1-2 hrs each at RT. Next, the cluster were exposed to 30% sucrose and tissue embedding medium (Leica) 1:1 overnight at 4°C. The clusters were transferred into an embedding mold and frozen by dry ice and stored at -80°C. The blocks were cut in 10 µm thick slices, dried for 10 min and stored at -20°C.

**Immunostainings of cryosections**

To rehydrate the cryosections, sections were washed 3 x with PBS, followed by permeabilization with 0.2% Triton X-100 and 0.1 M glycine in $H_2O$ for 15 minutes. Subsequently, sections were blocked (as described above) for 1-2 hours and then incubated in the primary antibody solution overnight at 4°C. The next day, the sections were rinsed 3 x and washed 3 x with PBST. The secondary antibody solution was added and kept for 3-5 hours at RT. Next, DAPI/PBS solution was added for 30 min, afterwards sections were rinsed and washed at least 3 x with PBST and mounted using Elvanol and dried overnight at RT.

**Protein isolation**

The cells were harvested and lysed in Radioimmunoprecipitation assay buffer (RIPA buffer: 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), Protease inhibitors) for 30 min on ice. If necessary, cells were sonicated for 15 seconds to completely lyse the cells. Next, the samples were spun down at 4°C and supernatant transferred into a clean tube and stored at -20°C for Western Blotting.

**Western Blotting**

Samples were thawed on ice and Laemmli buffer (4% SDS, 10% dithiothreitol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl, pH 6.8) was added. The samples were heated to 95°C for 5 min. After samples reached RT, the proteins were loaded on the prepared gels. Western Blotting was performed based on traditional protocols (Mahmood and Yang 2012).

**Gene targeting of mESCs via CRISPR/Cas9-system**

The Foxa2$^{Venus/Venus}$ KO and Snail1 KO mESCs lines were generated with the CRISPR/Cas9 gene targeting strategy. The gRNAs were designed via the online tool Optimized CRISPR Design (crispr.mit.edu/) and gRNAs were chosen based on their score values and their off-targets. The cloning strategy as well primer design were performed with the Clone Manager software (Sci Ed Software LLC).

**Cloning of targeting constructs**

Traditional cloning or Gibson assembly (NEB) was performed based on manufacturers recommendations. The constructs were transformed into competent DHα5F' bacteria.

**DNA extraction**

DNA extraction of transformed bacteria was performed with the QIAGEN Plasmid kits and followed the manufacturers protocol.

**Transfection of mESCs**

The DNA constructs were introduced into the mESCs by either Lipofectamine transfection (Thermo Fisher) or electroporation following the manufactures protocol. 48 hours after transfection, cells were selected with G418 and picked clones were expanded and analyzed by PCR genotyping.

**Preparation of genomic DNA from picked mESC clones**

DNA was extracted from the picked mESC clones by ethanol precipitation. In brief, cells were lysed (lysis buffer: 10 mM Tris, 10 mM EDTA, 10 mM NaCl, 0,5% (w/v) sarcosyl, 1 mg/ml proteinase K) at 60° overnight. The next day the DNA was precipitated by adding NaCl/Ethanol solution to each well and kept at RT for 30 min. The plates were carefully inverted to remove the NaCl/Ethanol solution. The DNA was washed three times with 70% ice-cold ethanol. After the final wash DNA was dissolved in TE buffer.

**Genotyping of picked mESC clones**

Picked mESC clones were genotyped by PCR following the NEB routine protocol. The PCR primers were designed with the Clone Manager software (Sci Ed Software LLC) binding upstream or downstream of the 5' or 3' end of the homology arms and inside the KI construct or WT sequence. Next, the recombination border of the targeting vector and WT sequence from the PCR product of the KI specific band were sequenced.

**Southern Blotting of mESC clones**

To exclude multiple insertions of the construct within the genome of the targeted mESCs Southern Blotting was performed as described previously (Southern 2006). Probe specific PCR primers were designed, and traditional PCR reaction performed to amplify the southern blot probe.

**RNA extraction and cDNA synthesis**

RNA was extracted by RNeasy Mini kit or miRNA micro kit (Qiagen) and cDNA synthesized by SuperScript VILO kit (Thermo Fisher). RNA extraction and cDNA synthesis were carried out according to the manufacturers protocol. RNA was stored at -80°C and cDNA at -20°C.

**Quantitative PCR (qPCR)**

Gene expression was assessed in differentiated cells by Taqman Arrays (Applied Biosystems) and data were analyzed using Expression Suite Software (Applied Biosystems) and normalized to undifferentiated hESCs using ΔΔCt method.

**Image analysis**

Images were taken with Leica SP5 and Zeiss LSM 880 Airy Scan confocal microscopes. Images acquired by Leica confocal were analyzed using Leica LAS AF Lite and images taken by Zeiss confocal microscope were processed using Zeiss Zen Blue software.

**Statistical analysis and reproducibility**

All values are depicted as means ±s.e.m. All statistical tests performed are mentioned in figure legends/methods for each data set. Statistical significance is defined as $P<0.05$. Comparison of 3 or more data sets was performed. All statistics were performed using GraphPad Prism software 8.

## 4 Publications for dissertation

Mahaddalkar P.U. *, **Scheibner K.***, Pfluger S., Ansarullah, Sterr M., Beckenbauer J., Irmler M., Beckers J., Knöbel S, Lickert H. **Generation of pancreatic β-cells from CD177$^{+}$ anterior definitive endoderm.** Nature Biotechnology. 2020, doi: 10.1038/s41587-020-0492-5 (*Co-first author).

**Summary.** The scarcity of human cadaveric islets provoked the search for an alternative cure of diabetes - namely, cell replacement therapy by stem cell-derived β-cells. However, current protocols produce mixed cell populations, consisting of endocrine cells but also pancreatic progenitor cells and non-pancreatic cells, resulting in immature SC-β-cells. To overcome this heterogeneity and to improve the quality of generated SC-β-cells, we performed a surface antibody screen and identified two surface antibodies that mark highly specified organ progenitors from seemingly homogenous endoderm as observed before from mouse fate-map studies. These organ progenitors are subpopulations of the ADE and receive differential canonical and noncanonical WNT signaling with a high differentiation potential towards either liver or pancreas fate. Differentiation of the pancreatic progenitor population towards β-cells revealed not only an increased number but also improved maturation and thus functionality of the generated SC-β-cells compared to unsorted cultures. Our novel approach to generate SC-β-cells is a step forward to generate a safe and efficient product for cell replacement therapy of type 1 diabetic patients.

**Declaration of contribution.** The differentiations of the H1 hESCs line towards SC-β-cells for functional analysis, such as dynamic and static GSIS, immunofluorescence stainings and FACS analysis were performed by me. The WNT inhibitor experiments with differentiated iPSCs were executed by me. Furthermore, I performed analysis of differentiations, such as immunostainings, FACS analysis, ELISA and qPCR. Differentiations of iPSC line were mainly performed by Pallavi Mahaddalkar. The surface antibody screen was done by Sebastian Knöbel. Sandra Pfluger performed differentiation experiments with H1 hESCs. Ansarullah performed the dynamic GSIS experiments. The manuscript was initially written by Pallavi Mahaddalkar and Heiko Lickert. Pallavi Mahaddalkar and I performed experiments and made changes in the figures design during the first revision. The second revision involving experiments and editing of the manuscript and figures was performed by me.

Bakhti, M. [+,] *, **Scheibner K.***, Tritschler S, Bastidas-Ponce A., Tarquis-Medina M., Theis F.J., Lickert H[+]. **Establishment of a high-resolution 3D modeling system for studying pancreatic epithelial cell biology *in vitro***. Molecular Metabolism. 2019 Sep 12; https://doi.org/10.1016/j.molmet.2019.09.005 (*Co-first author; [+]Co-corresponding).

**Summary.** Understanding the development of the human pancreas in health and disease is required to improve the generation of SC-endocrine cells for cell replacement therapy but also to discover novel therapeutic targets for diseases such as pancreatic cancer. Thus, we established an easy and reproducible 3D modeling system of cysts generated from human and mouse pancreatic progenitors. Mouse cysts were derived from embryonic pancreas, while human cysts derived from *in vitro* differentiated pancreatic progenitors of hiPSCs. The established culture condition consisting of Matrigel-coated surfaces and defined media allowed the survival, polarized cyst formation and endocrine differentiation of the pancreatic progenitors. The analysis of published scRNA-seq data sets of mouse and human pancreatic lineages revealed how apical-basal polarity, tight and adherence junctions change during the differentiation from a pancreatic progenitor to an endocrine cell. With our novel cyst system, we were able to confirm these changes during endocrinogenesis on a single-cell level in a high resolution. This 3D modeling system allows to monitor dynamic processes occurring during mouse and human pancreas development but also disease, thus providing a platform for numerous applications, such as improved differentiation protocols and drug discovery.

**Declaration of contribution.** The *in vitro* differentiations of hiPSCs and human cyst generation as well as immunostainings and FACS analysis was performed by me. Mostafa Bakhti generated the cysts derived from mouse pancreas and Sophie Tritschler reanalyzed the scRNA-seq data sets. The manuscript was written by Mostafa Bakhti and by me.

# 5 Discussion

Understanding the mechanisms involved in endoderm and endocrine lineage specification *in vivo* is essential for stem cell therapy and disease modeling. At present, the *in vitro* β-cell differentiation protocols produce a low quantity of SC-β-cells that are functionally immature. Furthermore, the analysis of dynamic processes during pancreas development and disease progression is impossible with the current human pancreatic modeling systems. Therefore, we aimed to analyze human endoderm heterogeneity, endoderm subtype potency and the signaling requirements for the induction of the pancreatic fate to eventually improve *in vitro* β-cell differentiation. Moreover, we established a 3D pancreatic modeling system that will help to understand conserved and species-specific mechanisms of human and mouse differentiation and morphogenesis but also to unravel pathomechanisms of pancreatic diseases for novel disease treatment strategies.

## 5.1 Novel approach to generate more mature and functional SC-β-cells

So far, diabetes can only be cured by the transplantation of cadaveric human islets, but their restricted availability, shifted the attention towards β-cell replacement therapies. Yet, the recent β-cell differentiation protocols give rise to heterogenous cell populations and immature SC-β-cells. Therefore, in this work we established a protocol to generate more functionally mature SC-β-cells by the enrichment of a highly specified progenitor population. A surface antibody screen of the definitive endoderm discovered two surface markers, CD177/NB1 glycoprotein and inducible T cell co-stimulatory ligand CD275/ICOSL, that mark pancreas and liver progenitors, respectively. The surface markers CD177 and CD275 identified subpopulations of the anterior definitive endoderm with differences in endoderm gene expression and inverse activation of canonical and noncanonical WNT signaling. The isolation of $CD177^+$ ADE cells and further differentiation resulted in a more efficient and homogenous generation of pancreatic progenitors and SC-β-cells. Moreover, the generated SC-β-cells expressed higher levels of maturation markers and were more glucose-responsive compared to unsorted SC-β-cells. Altogether, we analyzed human endoderm heterogeneity and identified a highly specified endoderm subpopulation with a defined signaling milieu showing an improved pancreatic differentiation potential and further differentiation generated SC-β-cells with superior functionality that resemble the endogenous counterpart.

### 5.1.1 Patterning and fate specification of human endoderm

Pancreatic endocrine cells, such as β-cells, derive from the ADE, thus the specification towards a specified anterior endoderm fate during *in vitro* differentiations is crucial for the generation of functional SC-β-cells. Currently it is unknown whether and how endodermal fates, such as anterior versus posterior endoderm, or organ progenitors of pancreas, liver or lung are specified due to the inaccessibility at this stage of human development (Jennings et al. 2013). Although several studies tried to induce different endodermal fates by modifying signaling pathways (Hsu et al. 2018; Yoney et al. 2018), the specification of defined organ progenitor populations has not been achieved. In this study, we provide for the first-time insights into the fine-tuned morphogen gradients required for the patterning and fate specification during human endoderm development *in vitro,* as observed before from mouse fate-map studies (Tam et al. 2006; Tremblay and Zaret 2005). To analyze if organ progenitors can already be identified in endoderm, we performed a screen with 330 surface antibodies, of which 30 marked subpopulations of the endoderm. This analysis highlighted the remarkable heterogeneity at DE stage during *in vitro* differentiations. We identified and focused on the surface antibodies CD177 and CD275, markers for pancreas and liver progenitors, respectively. Molecular profiling of the $CD177^+$- and $CD275^+$ ADE revealed differential expression of ADE genes and components of the canonical and noncanonical WNT signaling pathway. CER1, a Nodal, Bmp and Wnt signaling antagonist (Piccolo et al. 1999) was expressed in both $CD275^+$- and $CD177^+$ ADE, however at significantly higher levels in $CD177^+$ ADE. During mouse development, high TGFβ/Nodal activity promotes anterior fate, while lower Nodal- and Bmp signaling from the extra-embryonic region and high canonical Wnt/β-catenin signaling at the posterior side of the embryo specifies posterior endoderm fate (Zorn and Wells 2009). Together, this suggests an auto-regulatory feedback loop that modulates ligand–receptor interactions in these ADE cells, and thus regulates the signaling milieu essential to sustain anterior characteristic of the $CD275^+$- and $CD177^+$ ADE organ progenitors. Furthermore, canonical WNT signaling was down- and WNT/PCP signaling upregulated in $CD177^+$ ADE that induced the specification towards the pancreatic fate. In contrast, $CD275^+$ ADE upregulated the transcription factor HHEX and components of the canonical WNT signaling pathway that prompted a liver fate. This implies that $CD275^+$- and $CD177^+$ ADE organ progenitors are differentially patterned, which results in the specification of liver and pancreas organ domains and those are maintained by autoregulatory feedback mechanisms and signaling niches that lead to self-patterning (Sasai 2013). Self-organizing capabilities, including self-assembly, self-patterning and self-driven morphogenesis, of ESCs *in vitro* have been demonstrated previously by the generation of highly

ordered structures such as multilayered cortical tissues, neural retina and functional pituitary tissue.

Further studies are required to unravel whether modulating the signaling environment during endoderm formation can induce the expression of CD177 in endoderm progenitors to improve bulk differentiations. For instance, a transient inhibition of BMP and/or canonical WNT signaling or the stimulation of noncanonical WNT/PCP signaling during endoderm formation might promote the generation of $CD177^+$ ADE. Altogether, the characterization of $CD177^+$ and $CD275^+$ ADE subpopulations highlighted not only the heterogeneity of endoderm but also the differential intrinsic and extrinsic signals that are required for specification of organ progenitors. Thus, identifying signaling niches that are important for patterning of endoderm subpopulations will allow improved *in vitro* differentiations towards endoderm-derived organs such as the lung, thyroid and thymus.

### 5.1.2 CD177 a marker to predict pancreatic differentiation efficiency

*In vitro* differentiations must be robust and controlled independent of the genetic background of cell lines to allow utility and clinical safety of the stem cell derived products, such as prevention of teratoma formation, for cell replacement therapies (Latres et al. 2019). Furthermore, robust differentiations are also important to assure reproducible disease modeling (Volpato and Webber 2020). At present, every differentiation protocol induces endoderm differently with diverse cytokines, concentrations and treatment durations, but result in seemingly homogenous endoderm with similar percentages of pan-endoderm markers, such as CXCR4 (Hogrebe et al. 2020; Nair et al. 2019; Rezania et al. 2014; Velazco-Cruz et al. 2019). However, the different endoderm induction schemes will probably induce different patterning of the generated ADE subpopulations and thus give rise to other foregut-derived lineages, such as liver or lung, and impact the following differentiation towards pancreatic progenitors and endocrine cells. A correct patterning of ADE is essential to induce the specific organ progenitor population for improved differentiations and to reduce the amount of non-pancreatic cell types. Furthermore, the genetic background and non-genetic factors, such as clonal variation and the passage number, of the different hESC and hiPSC lines impact the efficiency of protocols (Bock et al. 2011). So far, it was not possible to isolate highly specified endoderm subpopulations for directed differentiations into endoderm-derived organs. However, our surface antibody screen showed that 30 surface antibodies distinguish subpopulations of the DE, demonstrating how heterogenous the endoderm is. The identification of CD177 and the other 30 endoderm markers could help to dissect endoderm heterogeneity and improve controlled and directed *in vitro*

differentiations in different genetic backgrounds. A thorough characterization of the endoderm subpopulations by a novel technology, namely Cellular Indexing of Transcriptomes and Epitopes by sequencing (CITE-seq) (Stoeckius et al. 2017), that connects the expression of epitopes and transcriptomes of a single cell, could uncover their fate. For instance, CD177 is transiently expressed, peaking at endoderm patterning (DE stage) and then gradually decreasing until organ lineage specification and determination (pancreatic progenitor stage). If the other identified DE subpopulations are analyzed by CITE-seq at DE stage and then at organ lineage specification, their fate and also the required signaling milieu to pattern and direct these progenitors towards their organ lineage, such as thymus, liver or lung, could be revealed.

Moreover, we observed that CD177 expression positively correlates with PDX1 induction at stage 3 of differentiation in several human ESC and iPSC lines. Thus, CD177 can be used as a prediction marker to determine the pancreatic differentiation potential at an early stage of differentiation independent of the genetic background of the cell line and endoderm induction schemes. Analyzing CD177 expression at the first step of differentiation will help to avoid unnecessary time consuming and costly differentiations if a certain percentage of the endoderm fails to express CD177. In addition, CD177 can be used to optimize different endoderm induction schemes for improved pancreatic differentiation for hPSC lines with different genetic background. Furthermore, endodermal progenitor cell lines generated from hPSCs have been established with self-renewal capacity and endodermal fate (Cheng et al. 2012). These endodermal progenitors were maintained as homogenous undifferentiated endoderm progenitors and upon stimulation differentiated into mainly mono-hormonal SC-β-cells, suggesting that a prolonged culture of endodermal cells might increase homogenous differentiations towards SC-β-cells. Thus, the isolation and expansion of $CD177^+$ ADE, and the differentiation from such highly specified and defined ADE could additionally improve the safety, robustness, yield and homogeneity of β-cell differentiations. However, further work is needed to test whether the expansion of $CD177^+$ ADE cells has an impact on their pancreatic differentiation potential.

### 5.1.3 Noncanonical WNT signaling specifies pancreatic progenitor formation

The canonical and noncanonical Wnt signaling pathways are repeatedly (in) activated from endoderm until pancreas formation (Scheibner et al. 2019). For instance, specification of pancreatic organ progenitors and later endocrine cells requires inhibition of canonical Wnt signaling and induction of noncanonical Wnt/PCP signaling (Cortijo et al. 2012; Rodríguez-Seguel et al. 2013). While the Wnt/β-catenin pathway mediates cell expansion and

differentiation, the noncanonical Wnt/PCP pathway regulates differentiation, cell dynamics, and morphogenesis important for tissue and organ architecture and eventually required for their function (Scheibner et al. 2019). The Wnt/PCP signaling pathway regulates cell polarity and tissue architecture through downstream effectors such as small GTPases and their targets like Rho-associate protein kinase (ROCK), that regulate the actin and microtubule cytoskeleton (Komiya and Habas 2008).

We observed that $CD177^+$ ADE receives noncanonical WNT/PCP signaling and is biased towards the pancreatic fate, while $CD275^+$ ADE shows activated canonical WNT signaling and is biased towards liver differentiation. To resemble the signaling environment of $CD177^+$ ADE cells for an improved pancreatic differentiation of unsorted cultures, we inhibited the secretion of WNT ligands or induced noncanonical WNT signaling resulting in increased pancreatic differentiation, while induction of canonical WNT signaling diminished pancreatic fate. This is in line with *in vivo* data from mouse showing differential Wnt signaling for liver versus pancreas fate (Rodríguez-Seguel et al. 2013). Although modulation of WNT signaling in unsorted differentiations can induce the pancreatic fate, contaminating cell types with a different fate, such as mesodermal cells, which might impact the specification and identity of endoderm-derived organ progenitors, are still in the culture. Moreover, apart from external signaling cues also the regulation of polarity, cytoskeleton, cell-cell adhesion, morphogenesis and mechanotransduction influence gene transcription and fate specification, however such complex conditions are restricted in mixed cell cultures (Bankaitis et al. 2018; Hogrebe et al. 2020; Löf-Öhlin et al. 2017; Mamidi et al. 2018). Thus, enrichment of specified $CD177^+$ organ progenitors depletes unwanted cell populations and provides a more homogenous and directed differentiation towards pancreatic lineages.

### 5.1.4 Enrichment of $CD177^+$ ADE improves the quantity of generated SC-β-cells and their functionality

The ultimate goal of β-cell replacement therapies is to generate β-cells that will safely restore normoglycemia independent of exogenous supply of insulin in T1DM patients. Current differentiation protocols (Hogrebe et al. 2020; Nair et al. 2019; Velazco-Cruz et al. 2019) produce SC-β-cells with improved functionality compared to previous protocols (Rezania et al. 2014), however they still lack a mature β-cell identity comparable to their counterpart in human islets. These protocols are performed as bulk differentiations using different morphogens, treatment durations and different geometric and mechanical influences that guide β-cell differentiation. However, pancreatic lineages derive from a highly specified ADE

subpopulation; and tightly regulated stage specific WNT signaling as well as coordinated morphogenesis are essential to form mature β-cells (Scheibner et al. 2019). We identified a $CD177^+$ pancreatic progenitor population in the ADE that highly expresses WNT/PCP signaling, while canonical WNT signaling is downregulated. Enrichment of $CD177^+$ ADE and further differentiation towards pancreatic progenitors revealed that CD177-derived pancreatic progenitors formed more defined, tight and compact clusters probably due to their high expression of WNT/PCP signaling, which coordinates actin cytoskeleton and that likely also modulates cell adhesion leading to increased cell-cell adhesion. Ultimately, the enrichment of highly specified organ progenitors together with a compact cluster formation resulted in more homogenous and mature SC-β-cells suggesting that cluster architecture and polarization have an impact on maturation and functionality. This is in line with findings in mouse, that Wnt/PCP signaling as well as islet architecture and compaction are important for insulin secretion of β-cells (Bader et al. 2016; Roscioni et al. 2016). Wnt/PCP signaling regulates the actin cytoskeleton, and tightly controlled insulin secretion requires the rearrangements of the actin cytoskeletal for trafficking of insulin granules through polarized microtubules from intracellular regions to the plasma membrane and following exocytosis (Kalwat and Thurmond 2013).

The maturation marker MAFA was expressed in significantly more CD177-derived SC-β-cells (CD177-β-cells), compared to unsorted SC-β-cells. So far, the induction of MAFA expression during human development is controversial. Immunohistochemical analysis of human islets discovered the first MAFA expressing cells at 4 years of age, while scRNA-seq analysis revealed its expression already during embryonic development (Cyphert et al. 2019; Ramond et al. 2017, 2018). However, we observed MAFA protein expression in CD177-β-cells, suggesting a rather embryonic or early postnatal induction of MAFA protein synthesis during human development. Furthermore, GLUT1, important for glucose uptake and glucose sensing to initiate insulin secretion, was highly expressed in CD177-β-cells. An increase in maturation markers was also reflected in enhanced functionality of CD177-β-cells. Upon static and dynamic glucose stimulations CD177-β-cells showed improved insulin secretion compared to unsorted SC-β-cells. Together this demonstrates that enrichment of specified organ progenitors results in a more homogenous differentiation generating more mature SC-β-cells with superior functionality compared to unsorted differentiations. Recently published differentiation protocols (Hogrebe et al. 2020; Nair et al. 2019; Velazco-Cruz et al. 2019) generated SC-β-cells that showed improved insulin secretion upon glucose stimulations compared to previous

protocols (Rezania et al. 2014). However, these protocols gave rise to SC-β-cells that lacked the protein synthesis of both maturation markers MAFA and GLUT1. In addition, scRNA-seq datasets of human islets and SC-β-cells pointed to overlaps but also discrepancies in the transcriptional profile of human islet β-cells and SC-β-cells (Veres et al. 2019). Collectively, this shows that although SC-β-cells generated by bulk differentiations can to some extend respond to glucose stimulations similar to islet β-cells, their transcriptional β-cell identity still differs to their endogenous counterpart. In contrast to bulk differentiations, CD177-β-cells expressed MAFA and GLUT1 suggesting a more similar transcriptional profile to islet β-cells, however this requires more experimental analysis. A possible explanation for the decreased β-cell maturation in bulk differentiations could be the presence of a significant fraction of non-pancreatic endocrine cells or progenitors (Sharon, Vanderhooft, et al. 2019). Currently, it is unknown how other lineages influence SC-β-cell development and functionality, however paracrine signals released from neighboring non-pancreatic cell types could negatively influence the maturation of β-cells. Certainly, the enrichment of pancreatic progenitors by the surface marker GP2 has proven to enhance differentiation efficiency and increased the purity and safety of the product (Ameri et al. 2017; Cogger et al. 2017). Thus, the enrichment of specified organ or pancreatic progenitor populations by CD177 or GP2 will not only improve differentiation efficiency but also decrease heterogeneity. In addition, differences in the differentiation protocols by (in) activation of pathways might lead to heterogeneity of cell populations and of the generated SC-β-cells. Therefore, surface antibodies such as CD177 and GP2 can serve as quality controls to detect if cells are on the right track during the differentiation. In addition, the isolation, expansion and storage of pancreatic progenitor populations by CD177 or GP2 can improve the robustness, safety and efficiency of SC-β-cell differentiations.

Although CD177-β-cells showed improved functionality compared to unsorted SC-β-cells, a tightly controlled glucose regulation, as observed in mature islet β-cells, is still missing. This is possibly due to the lack of other endocrine cells, such as α- and δ-cells, that are essential for controlled insulin secretion and dysfunctional in human diabetic islets (Gromada, Chabosseau, and Rutter 2018; Islam 2010).

At present, differentiation protocols focus on the generation of only SC-β-cells. Thus, approaches that aim to generate all endocrine cell types are of high interest for the future. Since CD177 marks progenitors of the pancreas, enrichment of $CD177^{+}$ organ progenitors together

with cell type specific differentiation protocols could be beneficial for the generation of other mature endocrine cells, like α- or δ-cells.

Finally, the objective of cell replacement therapy is the transplantation of functional and safe SC-β-cells or SC-islet-like clusters into T1DM patients. To date, the company ViaCyte developed the first and only cell replacement therapy using SC-derived pancreatic progenitors, which is currently tested in clinical trials for T1DM patients (www.viacyte.com). However, this approach bears the risk of uncontrolled differentiation towards ductal and acinar cells and tumor formation due to remaining pluripotent stem cells. A more defined and controlled approach could be achieved by using surface antibodies that only bind specific SC-endocrine cells and thus eliminate unwanted cells and furthermore allow a defined cluster composition. In recent years, several surface antibodies have been identified that are highly expressed in β-cells. For instance, CD49a was found to be highly enriched in SC-β-cells, however its expression was also observed in other hormone-expressing cells (Veres et al. 2019). Similarly, CD9 and ST8SIA1 are highly but not exclusively expressed in human pancreatic β-cells (Dorrell et al. 2016). Hence, extensive screenings of surface antibodies are necessary to identify markers that are exclusive to α-, β- and δ-cells. However, it is questionable if such exclusive surface markers can be identified, therefore combining several surface markers to enrich for a specific endocrine cell might be a more promising approach.

In addition, current differentiation protocols do not consider other cell types, such as mesenchymal and endothelial, that are present in human islets and important for maturation and function (Roscioni et al. 2016). Thus, mimicking the *in vivo* islet microenvironment *in vitro* will improve the generation of mature endocrine cells or SC-ILCs. Mature SC-ILCs that resemble the *in vivo* counterpart are of significance to allow disease modeling and drug screenings, but also for cell replacement therapies.

Thus, it is of highest importance to unravel the fine-tuned signaling milieu and morphogenetic processes that guide pluripotent stem cells towards completely functional endocrine cells. Altogether, this study broadened the understanding of human endoderm patterning and specification and furthermore demonstrated how enrichment of pancreatic progenitors provides a more defined and robust differentiation protocol resulting in more homogenous and functional SC-β-cells; thus moving one step closer to cell replacement therapy and disease modeling for diabetes.

## 5.2 High-resolution 3D system to model the pancreas *in vitro*

Although the current 3D *in vitro* differentiation protocols for the generation of SC-β-cells or SC-ILCs gained valuable insights into signaling requirements for the formation of pancreatic lineages, it is still unclear how expansion of pancreatic progenitors, induction of endocrine lineages, such as α- versus β-cells, or maturation of β-cells are achieved. Furthermore, disease modeling of pancreatic disorders, such as cancer and pancreatitis, are important for drug development and thus robust and reproducible *in vitro* modeling systems are needed. Together, more defined pancreatic modeling systems are required for the analysis of dynamic processes occurring during pancreas development as well as during pancreatic diseases on a single-cell level. Therefore, we generated a simple and reproducible 3D pancreatic cyst culture system to monitor conserved and species-specific processes during epithelialization and endocrinogenesis of the mouse and human pancreas *in vitro*. The system allowed us to monitor pancreatic progenitors during their differentiation into endocrine cells on a single-cell level in a high-resolution. The reanalysis of scRNA-seq data sets of mouse and human pancreatic lineages together with the cyst system revealed how polarity, cell-cell adhesion and transcription factors alter during endocrinogenesis. In summary, this *in vitro* modeling system provides a platform to not only study differentiation and morphogenesis of the pancreas but also disease development, progression and efficacy and safety of potential pharmaceutical treatments.

### 5.2.1 Generation of highly polarized mouse and human pancreatic cysts

Current challenges of cell replacement therapies are not only safety and functionality but also the quantity of SC-ILCs that are required for transplantation. Around 5000 islet equivalents (1 IEQ = ~150 µm)/kg body weight of the recipient must be transplanted to restore endogenous and physiologic insulin secretion in T1DM patients, thus large quantities of SC-ILCs have to be produced (Shapiro 2012). Therefore, unraveling mechanisms that involve expansion of pancreatic progenitors, but also efficient induction of endocrine cells are of high interest. During pancreas development, the multipotent pancreatic progenitors expand and eventually form a complex epithelial tubular network. Every epithelial branch harbors bipotent pancreatic progenitors that can differentiate into either endocrine lineages that delaminate from the duct or ductal cells (Bastidas-Ponce, Scheibner, et al. 2017). A comprehensive analysis of fine-tuned morphogen gradients and morphogenetic processes involved in the expansion of bipotent pancreatic progenitors and the specification of endocrine cells *in vivo* is limited in mouse and impossible in human, thus well-defined mouse and human modeling systems are needed (Bakhti, Böttcher, and Lickert 2019). Therefore, we established highly polarized 3D cyst

cultures of mouse and human pancreatic progenitors. Both mouse and human pancreatic cysts have the potential to differentiate into endocrine cells, showing that the pancreatic progenitors remained bipotent. A recently published pancreatic modeling system used mouse embryonic and human fetal pancreatic cells that were embedded in Matrigel and eventually formed large organoid structures that allowed pancreatic progenitor expansion and endocrine and acinar induction (Bonfanti et al. 2015). Our system does not mimic the complex pancreatic structure, consisting of pancreatic epithelial trunk and peripheral tip domains, but it resembles the pancreatic duct as a single-layered epithelium in well-defined small cysts. The cysts are not embedded in Matrigel, which allows live-cell imaging in a high resolution and the analysis of dynamic processes on a subcellular level. In addition, our human pancreas modeling system is based on differentiated hiPSCs and thus provides an unlimited source of human material for studies. Similarly, another pancreas modeling system used hESCs for disease modeling of the exocrine pancreas (Hohwieler et al. 2017). However, also this system generates large organoids that are covered by Matrigel, which impedes live-cell imaging and detailed analysis. Furthermore, this system was used to model the exocrine pancreas, but the endocrine differentiation potential was not tested. At present, our cyst system shows a low endocrine induction rate, however the other pancreatic modeling systems discussed here did not show endocrine induction efficiencies thus a comparison between our and the published modeling systems regarding endocrine differentiation potential is difficult. But compared to other 2D and 3D β-cell differentiation protocols, the endocrine induction efficiency of our system was lower (Rezania et al. 2014; Velazco-Cruz et al. 2019). However, these *in vitro* differentiation protocols do not fulfill the criteria required to model the pancreas. 2D systems lack the natural 3D structure of tissues that are important for cell orientation, polarity and differentiation and thus are limited for the analysis of processes such as morphogenesis and polarity dynamics. The 3D differentiation systems consist of aggregated cells, that fail to form a central lumen. This leads to disorganized cell clumps that do not resemble apical-basal polarity, cell-cell adhesion and cytoskeletal arrangements as observed *in vivo* during pancreas development.

In the future, our cyst system could be improved by adjusting for instance seeding densities, the duration and use of cytokines, more-define ECM components or glucose levels which could increase formation and maturation of endocrine cells (Hogrebe et al. 2020; Mamidi et al. 2018; Velazco-Cruz et al. 2019; Wang et al. 2020). In addition, using CD177-derived pancreatic progenitors for the 3D cyst culture system would improve endocrine differentiation and reveal novel insights into endocrinogenesis. Hence, the 3D cyst system is not yet an efficient system for pancreatic progenitor expansion or endocrine differentiation, but it offers a platform to

analyze early events of polarity establishment, pancreatic lumen formation, and endocrinogenesis in a time-resolved fashion on a single-cell level, which is not possible with the current *in vitro* modeling systems or *in vivo*.

### 5.2.2 Changes in TF expression during endocrinogenesis

Understanding the TF networks coordinating lineage priming, specification and determination towards an endocrine fate is essential for the generation of stem cell-derived endocrine cells. During endocrine induction Ngn3 is upregulated, followed by the expression of endocrine TFs such as NeuroD1, Nkx2.2 and Chromogranin A. Yet, the expression dynamics of TFs during endocrinogenesis have not been analyzed in detail in a time-resolved spatio-temporal pattern. Our pancreatic cyst system revealed that Foxa2 expression levels increase during endocrine differentiation and this is conserved in mouse and human pancreas development. In contrast, Pdx1 was increased during endocrine induction in mouse but not in human endocrinogenesis, indicating a species-specific TF activity during mouse and human endocrine differentiation. Similarly, Nkx2.2 shows a different expression pattern in human and mouse pancreas development (Jennings et al. 2013; Lyttle et al. 2008; Petri et al. 2006). During human pancreas development NKX2.2 is expressed in endocrine cells, while during mouse pancreas development Nkx2.2 is already observed in pancreatic progenitors. We observed that endocrine cells that highly express FOXA2 or endocrine markers, such as NKX2.2 or NEUROD1, are not attached to the apical lumen and instead were found in a second layer below the cyst epithelium. This finding could reflect the human endocrine delamination process consistent with observations in mouse cysts. Previously, it has been shown that the TFs Nkx2.2, Pdx1 and Foxa2 are not only important for β-cell formation but also involved in intact islet architecture and thus impact morphogenesis (Bastidas-Ponce et al. 2017; Doyle and Sussel 2007). Similar to our findings during endocrinogenesis, also during gastrulation, $Foxa2^{+}$ endoderm progenitors in the epiblast upregulate Foxa2 after their delamination (Burtscher and Lickert 2009). However, whether these TFs impact processes, such as cytoskeletal rearrangements, cell adhesion or polarity dynamics, that are important during delamination of endocrine cells requires further analyses.

### 5.2.3 Polarity and cytoskeletal dynamics during endocrinogenesis

The efficient generation of functional SC-ILCs is critical for cell replacement therapies and disease modeling. However, the mechanisms involved in endocrine cell induction and expansion are poorly understood, thus resulting in low endocrine induction efficiencies in β-cell differentiation protocols. During endocrinogenesis, bipotent pancreatic progenitors are highly polarized and arranged in a tube-like structure, following endocrine differentiation, the endocrine cells delaminate from the epithelial duct. Endocrine differentiation is impaired by the distraction of cell polarity of pancreatic progenitors (Kesavan et al. 2009). To understand polarity dynamics and their impact during endocrinogenesis thoroughly, we used our cyst system together with the analysis of published scRNA-seq data sets of human and mouse pancreatic lineages. We observed that mouse as well as human cysts expressed pancreatic progenitor and ductal markers, such as Foxa2, Pdx1 and Sox9. Furthermore, the generated cysts were highly polarized shown by a lumen formation and an apical domain enriched for F-actin and polarity proteins, such as aPKC and Ezrin, a finding consistent with *in vivo* mouse development but novel for human pancreas development (Villasenor et al. 2010). Upon endocrine differentiation these polarity proteins were downregulated. This is in line with previous findings showing that EGF signaling inhibits the apical polarity protein aPKC, which then results in apical narrowing in endocrine progenitors and their delamination (Löf-Öhlin et al. 2017). Cytoskeletal rearrangements then lead to blocking of Notch signaling and upregulation of Ngn3 to induce an endocrine fate. Moreover, we observed that ERM (ezrin-radixin-moesin)-binding phosphoprotein 50 (EBP50), an ERM binding and PDZ-scaffolding protein, was localized at the apical domain of pancreatic progenitors, which has not been shown before in mouse or human pancreas. In epithelial cells, EBP50 can form a complex with the ERM proteins and transmembrane proteins to connect the plasma membrane with F-actin (Vaquero et al. 2017). Studies in several cancer types have shown that EBP50 directly interacts with EGFR and furthermore loss of EBP50 in polarized cells results in a decrease of E-Cadherin from cell-cell junctions and polarity destruction (Vaquero et al. 2017). Interestingly, we noticed that upon endocrine induction EBP50 and E-Cadherin are transiently downregulated in human. The findings from our human cyst system and the scRNA-seq data sets together with recent observations indicate a possible scenario, where EGF signaling might degrade EBP50 and that leads to changes in E-Cadherin expression/redistribution and polarity loss, cytoskeletal rearrangements and endocrine induction, similar to previous observations (Löf-Öhlin et al. 2017). However, this hypothesis needs experimental proof and our cyst system could be used for such detailed analysis. Furthermore, Moesin, a member of the ERM family, and important

for cytoskeleton modifications and cell migration, was differentially expressed during mouse and human endocrinogenesis (Freymuth and Fitzsimons 2017). The expression of *Moesin* was increasing during human endocrinogenesis, while it was extremely low or absent during mouse development. Our data indicates that Moesin might be important for cytoskeletal rearrangements and migration during human endocrine induction and delamination but possibly dispensable for mouse development. However, further work is necessary to address the function of Moesin during mouse and human endocrinogenesis in more detail. Moreover, it was shown that non-muscle myosin II that acts on F-actin is essential for apical narrowing, basalward cell movement and Ngn3 upregulation, all regulated by ROCK activity (Bankaitis et al. 2018). ROCK is a downstream target of the noncanonical Wnt/PCP signaling pathway and it was shown that perturbations of Wnt/PCP signaling decrease endocrine differentiation (Cortijo et al. 2012). This indicates that Wnt/PCP signaling might be the activator of cytoskeletal changes important for endocrine differentiation, however this needs further experimental evidence. In summary, these findings implicate that extracellular signals, such as EGF, destruct apical-basal polarity and cytoskeletal rearrangements, coordinated by signaling cascades such as ROCK signaling, eventually lead to endocrine commitment and delamination. Our findings provide further evidence that apical-basal polarity is important for epithelial morphogenesis and upon endocrine induction polarity components are downregulated in mouse and human to allow endocrinogenesis. However, further studies are needed to understand how the polarity dynamics are rearranged after differentiation and delamination of endocrine cells. Overall, dynamic changes of cell polarity and the cytoskeleton are crucial for endocrine induction, consequently prospective β-cell differentiation protocols need to implement morphogens and/or physical and biomechanical cues that are important for an efficient differentiation of pancreatic progenitors into endocrine cells.

### 5.2.4 Cell adhesion rearrangements during endocrinogenesis

Understanding the fate decisions of bipotent pancreatic progenitors in the developing pancreas will advance the generation of endocrine cells from hPSCs for disease modeling approaches as well as cell replacement therapies. During endocrinogenesis, the apical-basal polarity of endocrine progenitors is disrupted leading to cellular rearrangements, such as remodeling and/or resolving of adherence and tight junctions. We observed that several cell-cell adhesion molecules were differentially expressed and distributed during endocrine induction. While pancreatic progenitors highly expressed E-Cadherin and β-Catenin, upon endocrine differentiation those proteins were downregulated or redistributed. Changes in adhesion

molecules have been shown to also impact cell differentiation by tissue segregation and cell sorting mechanisms (Townes and Holtfreter 1955). Indeed, a recent study showed that different expression levels of p120-catenin segregates trunk and tip domains but also α- versus β-cell fate (Nyeng et al. 2019). Similarly, also ECM-cell interactions have been shown to determine fate. Interaction of the ECM with integrin α5 induce a F-actin–YAP1–Notch signaling cascade that controls the fate of bipotent pancreatic progenitors (Mamidi et al. 2018). Based on these findings another study showed that actin depolymerization of the cytoskeleton is important for endocrine induction (Hogrebe et al. 2020). These studies together with our findings highlight a strong link between endocrine differentiation and rearrangements of cell-cell contacts, the cytoskeleton and cell-ECM interactions. Understanding the differential expression pattern of adhesion molecules and ECM components required to induce the endocrine lineage, will allow the efficient generation of endocrine cells from hPSCs. Therefore, our cyst system provides a platform to analyze in detail such complex processes, involving dynamic changes in cytoskeleton, polarity and distribution of cell surface proteins, by single-cell continuous live-cell imaging. For instance, Hogrebe et al. showed that actin depolymerization induces the endocrine lineage. The supplementation of a compound that influences cytoskeleton dynamics promoted the formation of β-cells over α- or δ-cells. This suggests that different levels of actin depolymerization might induce different endocrine cell types. Furthermore, the identification of cell surface proteins, such as integrins or cadherins, specific to endocrine cells would allow to enrich for those cells and thus would improve the directed differentiation towards endocrine cells. In summary, our novel 3D pancreatic modeling system offers a platform for a broad range of applications: studying human pancreas development and its translation for *in vitro* differentiations required for cell replacement therapy and disease modeling of endocrine and exocrine pancreatic disorders for drug screenings. Moreover, a more defined ECM environment and improved differentiation protocols will further enhance the application of this system for the expansion of pancreatic progenitors and endocrine cells for cell replacement therapies.

## 6 References


A., Pingitore et al. 2017. “Dynamic Profiling of Insulin Secretion and ATP Generation in Isolated Human and Mouse Islets Reveals Differential Glucose Sensitivity.” *Cellular Physiology and Biochemistry*.

Ahlgren, U., J. Jonsson, and H. Edlund. 1996. “The Morphogenesis of the Pancreatic Mesenchyme Is Uncoupled from That of the Pancreatic Epithelium in IPF1/PDX1-Deficient Mice.” *Development (Cambridge, England)* 122(5): 1409–16. http://www.ncbi.nlm.nih.gov/pubmed/8625829.

Ahlgren, Ulf et al. 1998. “β-Cell-Specific Inactivation of the Mouse Ipf1/Pdx1 Gene Results in Loss of the β-Cell Phenotype and Maturity Onset Diabetes.” *Genes and Development* 12(12): 1763–68.

Ameri, Jacqueline et al. 2017. “Efficient Generation of Glucose-Responsive Beta Cells from Isolated GP2+ Human Pancreatic Progenitors.” *Cell Reports*.

Ang, Siew Lan, and Janet Rossant. 1994. “HNF-3β Is Essential for Node and Notochord Formation in Mouse Development.” *Cell* 78(4): 561–74.

Apelqvist, Åsa et al. 1999. “Notch Signalling Controls Pancreatic Cell Differentiation.” *Nature* 400(August): 877–81.

Arda, H. Efsun et al. 2016. “Age-Dependent Pancreatic Gene Regulation Reveals Mechanisms Governing Human β Cell Function.” *Cell Metabolism*.

Arnold, Sebastian J., and Elizabeth J. Robertson. 2009. “Making a Commitment: Cell Lineage Allocation and Axis Patterning in the Early Mouse Embryo.” *Nature Reviews Molecular Cell Biology*.

Artner, Isabella et al. 2007. “MafB Is Required for Islet Beta Cell Maturation.” *Proceedings of the National Academy of Sciences of the United States of America* 104(16): 3853–58.

Asplound, K., S. Westman, and C. Hellersteöm. 1969. “Glucose Stimulation of Insulin Secretion from the Isolated Pancreas of Foetal and Newborn Rats.” *Diabetologia*.

Assady, Suheir et al. 2001. “Insulin Production by Human Embryonic Stem Cells.” *Diabetes*.

Bader, Erik et al. 2016. “Identification of Proliferative and Mature β-Cells in the Islets of Langerhans.” *Nature* 535(7612): 430–34.

Bakhti, Mostafa et al. 2019. “Establishment of a High-Resolution 3D Modeling System for Studying Pancreatic Epithelial Cell Biology in Vitro.” *Molecular Metabolism*.

Bakhti, Mostafa, Anika Böttcher, and Heiko Lickert. 2019. 15 Nature Reviews Endocrinology

*Modelling the Endocrine Pancreas in Health and Disease*. Springer US.

Bankaitis, Eric D. et al. 2018. "ROCK-Nmmyoii, Notch and Neurog3 Gene-Dosage Link Epithelial Morphogenesis with Cell Fate in the Pancreatic Endocrine-Progenitor Niche." *Development (Cambridge)* 145(18): dev162115.

Bankaitis, Eric D., Matthew E. Bechard, and Christopher V.E. E Wright. 2015. "Feedback Control of Growth, Differentiation, and Morphogenesis of Pancreatic Endocrine Progenitors in an Epithelial Plexus Niche." *Genes and Development* 29(20): 2203–16.

Bastidas-Ponce, Aimée, Sara S. Roscioni, et al. 2017. "Foxa2 and Pdx1 Cooperatively Regulate Postnatal Maturation of Pancreatic β-Cells." *Molecular Metabolism*.

Bastidas-Ponce, Aimée, Katharina Scheibner, Heiko Lickert, and Mostafa Bakhti. 2017. "Cellular and Molecular Mechanisms Coordinating Pancreas Development." *Development* 144(16): 2873–88.

Baumgartner, Brett K. et al. 2014. "Distinct Requirements for Beta-Catenin in Pancreatic Epithelial Growth and Patterning." *Developmental Biology* 391(1): 89–98.

Bechard, Matthew E. et al. 2016. "Precommitment Low-Level Neurog3 Expression Defines a Long-Lived Mitotic Endocrine-Biased Progenitor Pool That Drives Production of Endocrine-Committed Cells." *Genes & Development* 30(16): 1852–65.

Beddington, Rosa S.P., and Elizabeth J. Robertson. 1999. "Axis Development and Early Asymmetry in Mammals." *Cell*.

Bhushan, Anil et al. 2001. "Fgf10 Is Essential for Maintaining the Proliferative Capacity of Epithelial Progenitor Cells during Early Pancreatic Organogenesis." *Development (Cambridge, England)* 128(24): 5109–17.

Bock, Christoph et al. 2011. "Reference Maps of Human Es and Ips Cell Variation Enable High-Throughput Characterization of Pluripotent Cell Lines." *Cell*.

Bonfanti, Paola et al. 2015. "Ex Vivo Expansion and Differentiation of Human and Mouse Fetal Pancreatic Progenitors Are Modulated by Epidermal Growth Factor." *Stem Cells and Development* 24(15): 1766–78.

Bonner-Weir, Susan, Cristina Aguayo-Mazzucato, and Gordon C. Weir. 2016. "Dynamic Development of the Pancreas from Birth to Adulthood." *Upsala Journal of Medical Sciences*: 121, 155–58.

Bramswig, N. C., and K. H. Kaestner. 2011. 13 Diabetes, Obesity and Metabolism *Transcriptional Regulation of α-Cell Differentiation*.

Brissova, Marcela et al. 2002. "Reduction in Pancreatic Transcription Factor PDX-1 Impairs Glucose-Stimulated Insulin Secretion." *Journal of Biological Chemistry*.

Burlison, Jared S. et al. 2008. “Pdx-1 and Ptf1a Concurrently Determine Fate Specification of Pancreatic Multipotent Progenitor Cells.” *Developmental Biology*.

Burtscher, I., and H. Lickert. 2009. “Foxa2 Regulates Polarity and Epithelialization in the Endoderm Germ Layer of the Mouse Embryo.” *Development*.

Burtscher, Ingo et al. 2012. “The Sox17-MCherry Fusion Mouse Line Allows Visualization of Endoderm and Vascular Endothelial Development.” *Genesis*.

Burtscher, Ingo, Wenke Barkey, and Heiko Lickert. 2013. “Foxa2-Venus Fusion Reporter Mouse Line Allows Live-Cell Analysis of Endoderm-Derived Organ Formation.” *Genesis*.

Cabrera, Over et al. 2006. “The Unique Cytoarchitecture of Human Pancreatic Islets Has Implications for Islet Cell Function.” *Proceedings of the National Academy of Sciences of the United States of America*.

Cernilogar, Filippo M et al. 2019. “Pre-Marked Chromatin and Transcription Factor Co-Binding Shape the Pioneering Activity of Foxa2.” *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkz627.

Cheng, Xin et al. 2012. “Self-Renewing Endodermal Progenitor Lines Generated from Human Pluripotent Stem Cells.” *Cell Stem Cell*.

Churchill, Angela J. et al. 2017. “Genetic Evidence That Nkx2.2 Acts Primarily Downstream of Neurog3 in Pancreatic Endocrine Lineage Development.” *eLife*.

Cissell, Michelle A. et al. 2003. “Transcription Factor Occupancy of the Insulin Gene in Vivo: Evidence for Direct Regulation by Nkx2.2.” *Journal of Biological Chemistry*.

Cogger, Kathryn F. et al. 2017. “Glycoprotein 2 Is a Specific Cell Surface Marker of Human Pancreatic Progenitors.” *Nature Communications*.

Collombat, Patrick et al. 2003. “Opposing Actions of Arx and Pax4 in Endocrine Pancreas Development.” *Genes and Development*.

Conrad, Elizabeth et al. 2016. “The MAFB Transcription Factor Impacts Islet α-Cell Function in Rodents and Represents a Unique Signature of Primate Islet β-Cells.” *American Journal of Physiology - Endocrinology And Metabolism* 310(3): E91–102.

Cortijo, Cedric, Mathieu Gouzi, Fadel Tissir, and Anne Grapin-Botton. 2012. “Planar Cell Polarity Controls Pancreatic Beta Cell Differentiation and Glucose Homeostasis.” *Cell Reports* 2(6): 1593–1606.

Cyphert, Holly A. et al. 2019. “Examining How the MAFB Transcription Factor Affects Islet β-Cell Function Postnatally.” *Diabetes*.

D’Amour, Kevin A. et al. 2005. “Efficient Differentiation of Human Embryonic Stem Cells to Definitive Endoderm.” *Nature Biotechnology*.

DeFronzo, Ralph A. et al. 2015. “Type 2 Diabetes Mellitus.” *Nature Reviews Disease Primers*.

Dorrell, Craig et al. 2016. “Human Islets Contain Four Distinct Subtypes of β Cells.” *Nature Communications*.

Doyle, Michelle J., and Lori Sussel. 2007. “Nkx2.2 Regulates β-Cell Function in the Mature Islet.” *Diabetes*.

Engert, S. et al. 2013. “Wnt/ -Catenin Signalling Regulates Sox17 Expression and Is Essential for Organizer and Endoderm Formation in the Mouse.” *Development*.

Freymuth, Patrick S., and Helen L. Fitzsimons. 2017. “The ERM Protein Moesin Is Essential for Neuronal Morphogenesis and Long-Term Memory in Drosophila.” *Molecular Brain*.

Fu, Accalia, Chandra E. Eberhard, and Robert A. Screaton. 2013. “Role of AMPK in Pancreatic Beta Cell Function.” *Molecular and Cellular Endocrinology*.

Galceran, Juan et al. 1999. “Wnt3a(-/-) -like Phenotype and Limb Deficiency in Lef1(-/-)Tcf1(-/-) Mice.” *Genes and Development*.

Gao, Nan et al. 2007. “Foxa2 Controls Vesicle Docking and Insulin Secretion in Mature β Cells.” *Cell Metabolism*.

Gao, Tao et al. 2014. “Pdx1 Maintains β-Cell Identity and Function by Repressing an α-Cell Program.” *Cell Metabolism* 19(2): 259–71.

Gere-Becker, Maja B., Claudia Pommerenke, Thomas Lingner, and Tomas Pieler. 2018. “Retinoic Acid-Induced Expression of Hnf1b and Fzd4 Is Required for Pancreas Development in Xenopus Laevis.” *Development (Cambridge)* 145(12): dev161372.

Gittes, George K. 2009. “Developmental Biology of the Pancreas: A Comprehensive Review.” *Developmental Biology* 326(1): 4–35. http://dx.doi.org/10.1016/j.ydbio.2008.10.024.

Gradwohl, Gérard, Andrée Dierich, Marianne LeMeur, and François Guillemot. 2000. “Neurogenin3 Is Required for the Development of the Four Endocrine Cell Lineages of the Pancreas.” *Proceedings of the National Academy of Sciences of the United States of America* 97(4): 1607–11.

Greggio, Chiara et al. 2013. “Artificial Three-Dimensional Niches Deconstruct Pancreas Development in Vitro.” *Development (Cambridge)* 140(21): 4452–62.

Gromada, Jesper, Pauline Chabosseau, and Guy A. Rutter. 2018. “The α-Cell in Diabetes Mellitus.” *Nature Reviews Endocrinology*.

Gu, Chunyan et al. 2010. “Pancreatic β Cells Require NeuroD to Achieve and Maintain Functional Maturity.” *Cell Metabolism*.

Gu, Guoqiang, Jolanta Dubauskaite, and Douglas A. Melton. 2002. “Direct Evidence for the Pancreatic Lineage: NGN3+ Cells Are Islet Progenitors and Are Distinct from Duct

Progenitors." *Development (Cambridge, England)* 129(10): 2447–57. http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=11973276&retmode=ref&cmd=prlinks.

Haegel, H et al. 1995. "Lack of β-Catenin Affects Mouse Development at Gastrulation." *Development*.

Hart, Alan, Stella Papadopoulou, and Helena Edlund. 2003. "Fgf10 Maintains Notch Activation, Stimulates Proliferation, and Blocks Differentiation of Pancreatic Epithelial Cells." *Developmental Dynamics* 228(2): 185–93.

Hauge-Evans, Astrid C. et al. 2009. "Somatostatin Secreted by Islet δ-Cells Fulfills MultipleRoles as a Paracrine Regulator of Islet Function." *Diabetes*.

Hebrok, Matthias et al. 2000. "Regulation of Pancreas Development by Hedgehog Signaling." *Development* 127: 4905–13. http://dev.biologists.org/content/127/22/4905.short.

Hebrok, Matthias, Seung K. Kim, and Douglas A. Melton. 1998. "Notochord Repression of Endodermal Sonic Hedgehog Permits Pancreas Development." *Genes and Development* 12(11): 1705–13.

Helman Aharon, Cangelosi Andrew L., Davis Jeffrey C., Pham Quan, Rothman Arielle, Faust Aubrey L., Straubhaar Juerg R., Sabatini, David M., Melton Douglas A. 2020. "A Nutrient-Sensing Transition at Birth Triggers Glucose-Responsive Insulin Secretion." *Cell Metabolism* 31(5): 1004–16.

Hitz, Christiane, Wolfgang Wurst, and Ralf Kühn. 2007. "Conditional Brain-Specific Knockdown of MAPK Using Cre/LoxP Regulated RNA Interference." *Nucleic Acids Research*.

Hogrebe, Nathaniel J. et al. 2020. "Targeting the Cytoskeleton to Direct Pancreatic Differentiation of Human Pluripotent Stem Cells." *Nature Biotechnology*.

Hohwieler, Meike et al. 2017. "Human Pluripotent Stem Cell-Derived Acinar/Ductal Organoids Generate Human Pancreas upon Orthotopic Transplantation and Allow Disease Modelling." *Gut*.

Hsu, Hui Ting, Conchi Estarás, Ling Huang, and Katherine A. Jones. 2018. "Specifying the Anterior Primitive Streak by Modulating YAP1 Levels in Human Pluripotent Stem Cells." *Stem Cell Reports*.

Huch, Meritxell, and Bon Kyoung Koo. 2015. "Modeling Mouse and Human Development Using Organoid Cultures." *Development (Cambridge)*.

International Diabetes Foundation. 2019. "International Diabetes Federation - Facts & Figures." *2019*.

Islam, Md Shadidul. 2010. “The Islets of Langerhans. Preface.” *Advances in experimental medicine and biology*.

James Shapiro, A. M. 2012. “Islet Transplantation in Type 1 Diabetes: Ongoing Challenges, Refined Procedures, and Long-Term Outcome.” *Review of Diabetic Studies*.

Jennings, Rachel E. et al. 2013. “Development of the Human Pancreas from Foregut to Endocrine Commitment.” *Diabetes* 62(10): 3514–22.

———. 2017. “Laser Capture and Deep Sequencing Reveals the Transcriptomic Programmes Regulating the Onset of Pancreas and Liver Differentiation in Human Embryos.” *Stem Cell Reports*.

Jensen, J et al. 2000. “Control of Endodermal Endocrine Development by Hes-1.” *Nature genetics* 24(1): 36–44.

Jiang, Wei et al. 2011. “CD24: A Novel Surface Marker for PDX1-Positive Pancreatic Progenitors Derived from Human Embryonic Stem Cells.” *Stem Cells*.

Jonsson, J, L Carlsson, T Edlund, and H Edlund. 1994. “Insulin-Promoter-Factor 1 Is Required for Pancreas Development in Mice.” *Nature* 371(6498): 606–9.

Kalwat, Michael A., and Debbie C. Thurmond. 2013. “Signaling Mechanisms of Glucose-Induced F-Actin Remodeling in Pancreatic Islet β Cells.” *Experimental and Molecular Medicine*.

Kaneto, Hideaki et al. 2009. “Role of MafA in Pancreatic β-Cells.” *Advanced Drug Delivery Reviews*.

Katsarou, Anastasia et al. 2017. “Type 1 Diabetes Mellitus.” *Nature Reviews Disease Primers*.

Kawaguchi, Yoshiya et al. 2002. “The Role of the Transcriptional Regulator Ptf1a in Converting Intestinal to Pancreatic Progenitors.” *Nature Genetics* 32(1): 128–34. http://www.nature.com/doifinder/10.1038/ng959.

Kelly, Olivia G. et al. 2011. “Cell-Surface Markers for the Isolation of Pancreatic Cell Types Derived from Human Embryonic Stem Cells.” *Nature Biotechnology*.

Kesavan, Gokul et al. 2009. “Cdc42-Mediated Tubulogenesis Controls Cell Specification.” *Cell* 139(4): 791–801.

Kim, Seung K. et al. 2000. “Activin Receptor Patterning of Foregut Organogenesis.” *Genes and Development* 14(15): 1866–71.

Kojima, Shinya et al. 2007. “A Role for Pancreatic Polypeptide in Feeding and Body Weight Regulation.” *Peptides*.

Komiya, Yuko, and Raymond Habas. 2008. “Wnt Signal Transduction Pathways.” *Organogenesis*.

Krentz, Nicole A.J. et al. 2017. “Phosphorylation of NEUROG3 Links Endocrine Differentiation to the Cell Cycle in Pancreatic Progenitors.” *Developmental Cell*.

Kumar, Maya, Nathalie Jordan, Doug Melton, and Anne Grapin-Botton. 2003. “Signals from Lateral Plate Mesoderm Instruct Endoderm toward a Pancreatic Fate.” *Developmental Biology*.

Kunisada, Yuya, Noriko Tsubooka-Yamazoe, Masanobu Shoji, and Masaki Hosoya. 2012. “Small Molecules Induce Efficient Differentiation into Insulin-Producing Cells from Human Induced Pluripotent Stem Cells.” *Stem Cell Research*.

Kwon, Gloria S., Manuel Viotti, and Anna Katerina Hadjantonakis. 2008. “The Endoderm of the Mouse Embryo Arises by Dynamic Widespread Intercalation of Embryonic and Extraembryonic Lineages.” *Developmental Cell*.

Lancaster, Madeline A., and Juergen A. Knoblich. 2014. “Organogenesisin a Dish: Modeling Development and Disease Using Organoid Technologies.” *Science*.

Larsen, Brian M et al. 2015. “Mesenchymal Hox6 Function Is Required for Pancreatic Endocrine Cell Differentiation.” *Development (Cambridge, England)* 142(22): 3859–68.

Latres, Esther et al. 2019. “Navigating Two Roads to Glucose Normalization in Diabetes: Automated Insulin Delivery Devices and Cell Therapy.” *Cell Metabolism*.

Lee, Jane C. et al. 2001. “Regulation of the Pancreatic Pro-Endocrine Gene Neurogenin3.” *Diabetes* 50(5): 928–36.

Lewis, Samara L., and Patrick P.L. Tam. 2006. “Definitive Endoderm of the Mouse Embryo: Formation, Cell Fates, and Morphogenetic Function.” *Developmental Dynamics*.

Lickert, Heiko et al. 2002. “Formation of Multiple Hearts in Mice Following Deletion of β-Catenin in the Embryonic Endoderm.” *Developmental Cell*.

Liu, Pentao et al. 1999. “Requirement for Wnt3 in Vertebrate Axis Formation.” *Nature Genetics*.

Löf-Öhlin, Zarah M. et al. 2017. “EGFR Signalling Controls Cellular Fate and Pancreatic Organogenesis by Regulating Apicobasal Polarity.” *Nature Cell Biology* 19(11): 1313–25.

Lyttle, B. M. et al. 2008. “Transcription Factor Expression in the Developing Human Fetal Endocrine Pancreas.” *Diabetologia* 51(7): 1169–80.

Magenheim, Judith et al. 2011. “Ngn3+ Endocrine Progenitor Cells Control the Fate and Morphogenesis of Pancreatic Ductal Epithelium.” *Developmental Biology*.

Mahmood, Tahrin, and Ping Chang Yang. 2012. “Western Blot: Technique, Theory, and Trouble Shooting.” *North American Journal of Medical Sciences*.

Mamidi, Anant et al. 2018. 564 Nature *Mechanosignalling via Integrins Directs Fate Decisions*

*of Pancreatic Progenitors*.

Martín, Mercè et al. 2005. "Dorsal Pancreas Agenesis in Retinoic Acid-Deficient Raldh2 Mutant Mice." *Developmental Biology* 284(2): 399–411.

Marty-Santos, L., and O. Cleaver. 2015. "Progenitor Epithelium: Sorting Out Pancreatic Lineages." *Journal of Histochemistry & Cytochemistry* 63(8): 559–74.

Mastracci, Teresa L., Keith R. Anderson, James B. Papizan, and Lori Sussel. 2013. "Regulation of Neurod1 Contributes to the Lineage Potential of Neurogenin3+ Endocrine Precursor Cells in the Pancreas." *PLoS Genetics* 9(2): 1–14.

Matschinsky, Franz M., and David F. Wilson. 2019. "The Central Role of Glucokinase in Glucose Homeostasis: A Perspective 50 Years after Demonstrating the Presence of the Enzyme in Islets of Langerhans." *Frontiers in Physiology*.

McCulloch, Laura J. et al. 2011. "GLUT2 (SLC2A2) Is Not the Principal Glucose Transporter in Human Pancreatic Beta Cells: Implications for Understanding Genetic Association Signals at This Locus." *Molecular Genetics and Metabolism*.

McGrath, Patrick S. et al. 2015. "The Basic Helix-Loop-Helix Transcription Factor Neurog3 Is Required for Development of the Human Endocrine Pancreas." *Diabetes*.

McLin, V. A., S. A. Rankin, and A. M. Zorn. 2007. "Repression of Wnt/ -Catenin Signaling in the Anterior Endoderm Is Essential for Liver and Pancreas Development." *Development* 134(12): 2207–17.

van der Meulen, Talitha et al. 2012. "Urocortin 3 Marks Mature Human Primary and Embryonic Stem Cell-Derived Pancreatic Alpha and Beta Cells." *PLoS ONE*.

Mfopou, Josué Kunjom et al. 2014. "Efficient Definitive Endoderm Induction from Mouse Embryonic Stem Cell Adherent Cultures: A Rapid Screening Model for Differentiation Studies." *Stem Cell Research*.

Miralles, Francisco, Paul Czernichow, and Raphael Scharfmann. 1998. "Follistatin Regulates the Relative Proportions of Endocrine versus Exocrine Tissue during Pancreatic Development." *Development* 125(0950-1991 (Print)): 1017–24.

Moates, J. Michael et al. 2003. "BETA2 Activates Transcription from the Upstream Glucokinase Gene Promoter in Islet β-Cells and Gut Endocrine Cells." *Diabetes*.

Müller, T. D. et al. 2015. "Ghrelin." *Molecular Metabolism*.

Muñoz-Bravo, Jose Luis et al. 2016. "Loss of Pancreas upon Activated Wnt Signaling Is Concomitant with Emergence of Gastrointestinal Identity." *PLoS ONE* 11(10): 1–22.

Murphy, Rinki, Sian Ellard, and Andrew T. Hattersley. 2008. "Clinical Implications of a Molecular Genetic Classification of Monogenic β-Cell Diabetes." *Nature Clinical*

*Practice Endocrinology and Metabolism*.

Nair, Gopika G. et al. 2019. "Recapitulating Endocrine Cell Clustering in Culture Promotes Maturation of Human Stem-Cell-Derived β Cells." *Nature Cell Biology*.

Nelson, Shelley B, Ashleigh E Schaffer, and Maike Sander. 2007. "The Transcription Factors Nkx6.1 and Nkx6.2 Possess Equivalent Activities in Promoting Beta-Cell Fate Specification in Pdx1+ Pancreatic Progenitor Cells." *Development (Cambridge, England)* 134(13): 2491–2500.

Nishimura, Wataru et al. 2006. "A Switch from MafB to MafA Expression Accompanies Differentiation to Pancreatic β-Cells." *Developmental Biology* 293(2): 526–39.

Nishimura, Wataru, Susan Bonner-Weir, and Arun Sharma. 2009. "Expression of MafA in Pancreatic Progenitors Is Detrimental for Pancreatic Development." *Developmental Biology*.

Norgaard, Gitte Anker, Jan Nygaard Jensen, and Jan Nygaard Jensen. 2003. "FGF10 Signaling Maintains the Pancreatic Progenitor Cell State Revealing a Novel Role of Notch in Organ Development." *Developmental Biology* 264(2): 323–38.

Nowotschin, Sonja et al. 2019. "The Emergent Landscape of the Mouse Gut Endoderm at Single-Cell Resolution." *Nature*.

Nowotschin, Sonja, Anna-Katerina Katerina Hadjantonakis, and Kyra Campbell. 2019. "The Endoderm: A Divergent Cell Lineage with Many Commonalities." *Development (Cambridge, England)*.

Nyeng, Pia et al. 2019. "P120ctn-Mediated Organ Patterning Precedes and Determines Pancreatic Progenitor Fate." *Developmental Cell* 49: 31–47.

Offield, M F et al. 1996. "PDX-1 Is Required for Pancreatic Outgrowth and Differentiation of the Rostral Duodenum." *Development (Cambridge, England)* 122(3): 983–95. http://www.ncbi.nlm.nih.gov/pubmed/8631275.

Pagliuca, Felicia W. et al. 2014. "Generation of Functional Human Pancreatic Beta Cells in Vitro." *Cell* 159(2): 428–39.

Pan, Fong Cheng, and Marcela Brissova. 2014. 21 Current Opinion in Endocrinology, Diabetes and Obesity *Pancreas Development in Humans*.

Pan, Fong Cheng, and Chris Wright. 2011. "Pancreas Organogenesis: From Bud to Plexus to Gland." *Developmental Dynamics* 240(3): 530–65.

Papizan, James B. et al. 2011. "Nkx2.2 Repressor Complex Regulates Islet β-Cell Specification and Prevents β-to-α-Cell Reprogramming." *Genes and Development*.

Petri, Andreas et al. 2006. "The Effect of Neurogenin3 Deficiency on Pancreatic Gene

Expression in Embryonic Mice." *Journal of Molecular Endocrinology* 37(2): 301–16.

Piccolo, Stefano et al. 1999. "The Head Inducer Cerberus Is a Multifunctional Antagonist of Nodal, BMP and Wnt Signals." *Nature*.

Pierreux, Christophe E. et al. 2010. "Epithelial: Endothelial Cross-Talk Regulates Exocrine Differentiation in Developing Pancreas." *Developmental Biology* 347(1): 216–27.

Pijuan-Sala, Blanca et al. 2019. "A Single-Cell Molecular Map of Mouse Gastrulation and Early Organogenesis." *Nature*. https://doi.org/10.1038/s41586-019-0933-9.

Piper, K. et al. 2004. "Beta Cell Differentiation during Early Human Pancreas Development." *Journal of Endocrinology*.

Polak, Michel, Linda Bouchareb-Banaei, Raphael Scharfmann, and Paul Czernichow. 2000. "Early Pattern of Differentiation in the Human Pancreas." *Diabetes*.

Qu, Xiaoling et al. 2013. "Notch-Mediated Post-Translational Control of Ngn3 Protein Stability Regulates Pancreatic Patterning and Cell Fate Commitment." *Developmental Biology* 376(1): 1–12.

Ramond, Cyrille et al. 2017. "Reconstructing Human Pancreatic Differentiation by Mapping Specific Cell Populations during Development." *eLife*.

———. 2018. "Understanding Human Fetal Pancreas Development Using Subpopulation Sorting, RNA Sequencing and Single-Cell Profiling." *Development (Cambridge)*.

Rezania, Alireza et al. 2014. "Reversal of Diabetes with Insulin-Producing Cells Derived in Vitro from Human Pluripotent Stem Cells." *Nature Biotechnology* 32(11): 1121–33.

Riedel, M. J. et al. 2012. "Immunohistochemical Characterisation of Cells Co-Producing Insulin and Glucagon in the Developing Human Pancreas." *Diabetologia* 55(2): 372–81.

Rivera-Pérez, Jaime A., and Anna Katerina Hadjantonakis. 2015. "The Dynamics of Morphogenesis in the Early Mouse Embryo." *Cold Spring Harbor Perspectives in Biology*.

Rodríguez-Seguel, Elisa et al. 2013. "Mutually Exclusive Signaling Signatures Define the Hepatic and Pancreatic Progenitor Cell Lineage Divergence." *Genes and Development* 27(17): 1932–46.

Rorsman, P., and E. Renström. 2003. "Insulin Granule Dynamics in Pancreatic Beta Cells." *Diabetologia*.

Roscioni, Sara S., Adriana Migliorini, Moritz Gegg, and Heiko Lickert. 2016. 12 Nature Reviews Endocrinology *Impact of Islet Architecture on β-Cell Heterogeneity, Plasticity and Function*. Nature Publishing Group.

Rossi, J. M., N. R. Dunn, B. L.M. Hogan, and K. S. Zaret. 2001. "Distinct Mesodermal Signals,

Including BMPs from the Septum, Transversum Mesenchyme, Are Required in Combination for Hepatogenesis from the Endoderm." *Genes and Development*.

Salinno, Ciro et al. 2019. "β-Cell Maturation and Identity in Health and Disease." *International Journal of Molecular Sciences*.

Sander, M et al. 2000. "Homeobox Gene Nkx6.1 Lies Downstream of Nkx2.2 in the Major Pathway of Beta-Cell Formation in the Pancreas." *Development* 127(24): 5533–40.

Sarkar, S. A. et al. 2008. "Global Gene Expression Profiling and Histochemical Analysis of the Developing Human Fetal Pancreas." *Diabetologia*.

Sasai, Yoshiki. 2013. "Cytosystems Dynamics in Self-Organization of Tissue Architecture." *Nature*.

Scheibner, Katharina, Mostafa Bakhti, Aimée Bastidas-Ponce, and H. Lickert. 2019. "Wnt Signaling: Implications in Endoderm Development and Pancreas Organogenesis." *Current Opinion in Cell Biology*.

Schwitzgebel, V. M. et al. 2000. "Expression of Neurogenin3 Reveals an Islet Cell Precursor Population in the Pancreas." *Development*.

Serls, Amanda E. et al. 2005. "Different Thresholds of Fibroblast Growth Factors Pattern the Ventral Foregut into Liver and Lung." *Development*.

Sharon, Nadav, Raghav Chawla, et al. 2019. "A Peninsular Structure Coordinates Asynchronous Differentiation with Morphogenesis to Generate Pancreatic Islets." *Cell* 176(4): 790-804.e13.

Sharon, Nadav, Jordan Vanderhooft, et al. 2019. "Wnt Signaling Separates the Progenitor and Endocrine Compartments during Pancreas Development." *Cell Reports*.

Shih, Hung Ping et al. 2012. "A Notch-Dependent Molecular Circuitry Initiates Pancreatic Endocrine and Ductal Cell Differentiation." *Development (Cambridge)* 139(14): 2488–99.

Sinagoga, Katie L. et al. 2017. "Distinct Roles for the MTOR Pathway in Postnatal Morphogenesis, Maturation and Function of Pancreatic Islets." *Development (Cambridge)*.

Slack, J. M.W. 1995. "Developmental Biology of the Pancreas." *Development*.

Solar, Myriam et al. 2009. "Pancreatic Exocrine Duct Cells Give Rise to Insulin-Producing ?? Cells during Embryogenesis but Not after Birth." *Developmental Cell* 17(6): 849–60.

Southern, Ed. 2006. "Southern Blotting." *Nature Protocols*.

Stoeckius, Marlon et al. 2017. "Simultaneous Epitope and Transcriptome Measurement in Single Cells." *Nature Methods*.

Stoffers, D. A., J. Ferrer, W. L. Clarke, and J. F. Habener. 1997. 17 Nature genetics *Early-Onset*

*Type-II Diabetes Mellitus (MODY4) Linked to IPF1*.

Sugiyama, Takuya et al. 2013. "Reconstituting Pancreas Development from Purified Progenitor Cells Reveals Genes Essential for Islet Differentiation." *Proceedings of the National Academy of Sciences of the United States of America*.

Sussel, L. et al. 1998. "Mice Lacking the Homeodomain Transcription Factor Nkx2.2 Have Diabetes Due to Arrested Differentiation of Pancreatic β Cells." *Development*.

Takaoka, K., and H. Hamada. 2011. "Cell Fate Decisions and Axis Determination in the Early Mouse Embryo." *Development*.

Tam, P. P. L., and R. S. P. Beddington. 2007. "Establishment and Organization of Germ Layers in the Gastrulating Mouse Embryo." In *Ciba Found Symp, ,* 27–41.

Tam, P P, and R S Beddington. 1992. "Establishment and Organization of Germ Layers in the Gastrulating Mouse Embryo." *Ciba Found Symp* 165: 27–41.

Tam, Patrick P.L., and Richard R. Behringer. 1997. "Mouse Gastrulation: The Formation of a Mammalian Body Plan." *Mechanisms of Development*.

Tam, Patrick P.L. L. et al. 2006. "Sequential Allocation and Global Pattern of Movement of the Definitive Endoderm in the Mouse Embryo during Gastrulation." *Development*.

Townes, Philip L., and Johannes Holtfreter. 1955. "Directed Movements and Selective Adhesion of Embryonic Amphibian Cells." *Journal of Experimental Zoology*.

Tremblay, Kimberly D., and Kenneth S. Zaret. 2005. "Distinct Populations of Endoderm Cells Converge to Generate the Embryonic Liver Bud and Ventral Foregut Tissues." *Developmental Biology* 280(1): 87–99.

Tschop, M., D. L. Smiley, and M. L. Heiman. 2000. "Ghrelin Induces Adiposity in Rodents." *Nature*.

Uc, Aliye, and Douglas S. Fishman. 2017. "Pancreatic Disorders." *Pediatric Clinics of North America*.

Vaquero, J., T. H. Nguyen Ho-Bouldoires, A. Clapéron, and L. Fouassier. 2017. "Role of the PDZ-Scaffold Protein NHERF1/EBP50 in Cancer Biology: From Signaling Regulation to Clinical Relevance." *Oncogene*.

Velazco-Cruz, Leonardo et al. 2019. "Acquisition of Dynamic Function in Human Stem Cell-Derived β Cells." *Stem Cell Reports*.

Veres, Adrian et al. 2019. "Charting Cellular Identity during Human in Vitro β-Cell Differentiation." *Nature*.

Villasenor, Alethia, Diana C. Chong, Mark Henkemeyer, and Ondine Cleaver. 2010. "Epithelial Dynamics of Pancreatic Branching Morphogenesis." *Development (Cambridge, England)*

137(24): 4295–4305. http://www.ncbi.nlm.nih.gov/pubmed/21098570%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2990215.

Viotti, Manuel, Sonja Nowotschin, and Anna Katerina Hadjantonakis. 2014. "SOX17 Links Gut Endoderm Morphogenesis and Germ Layer Segregation." *Nature Cell Biology*.

Volpato, Viola, and Caleb Webber. 2020. "Addressing Variability in IPSC-Derived Models of Human Disease: Guidelines to Promote Reproducibility." *DMM Disease Models and Mechanisms*.

De Vos, Anick et al. 1995. "Human and Rat Beta Cells Differ in Glucose Transporter but Not in Glucokinase Gene Expression." *Journal of Clinical Investigation*.

Wang, Daisong et al. 2020. "Long-Term Expansion of Pancreatic Islet Organoids from Resident Procr+ Progenitors." *Cell*.

Wang, Sui et al. 2010. "Neurog3 Gene Dosage Regulates Allocation of Endocrine and Exocrine Cell Fates in the Developing Mouse Pancreas." *Developmental Biology*.

Wang, Xianming et al. 2016. "Generation of a Human Induced Pluripotent Stem Cell (IPSC) Line from a Patient with Family History of Diabetes Carrying a C18R Mutation in the PDX1 Gene." *Stem Cell Research*.

Wang, Zengxin, Pascal Dollé, Wellington V. Cardoso, and Karen Niederreither. 2006. "Retinoic Acid Regulates Morphogenesis and Patterning of Posterior Foregut Derivatives." *Developmental Biology*.

Weedon, Michael N et al. 2013. "Recessive Mutations in a Distal PTF1A Enhancer Cause Isolated Pancreatic Agenesis." *Nature Publishing Group* 46(1): 61–64.

Weinstein, Daniel C. et al. 1994. "The Winged-Helix Transcription Factor HNF-3β Is Required for Notochord Development in the Mouse Embryo." *Cell* 78(4): 575–88.

Wessells, Norman K., and Julia H. Cohen. 1967. "Early Pancreas Organogenesis: Morphogenesis, Tissue Interactions, and Mass Effects." *Developmental Biology* 15(3): 237–70. http://www.sciencedirect.com/science/article/pii/0012160667900425.

Ye, F., B. Duvillié, and R. Scharfmann. 2005. "Fibroblast Growth Factors 7 and 10 Are Expressed in the Human Embryonic Pancreatic Mesenchyme and Promote the Proliferation of Embryonic Pancreatic Epithelial Cells." *Diabetologia*.

Yoney, Anna et al. 2018. "WNT Signaling Memory Is Required for ACTIVIN to Function as a Morphogen in Human Gastruloids." *eLife*.

Yoshihara, Eiji et al. 2016. "ERRγ Is Required for the Metabolic Maturation of Therapeutically Functional Glucose-Responsive β Cells." *Cell Metabolism*.

Zaret, Kenneth S. 2008. “Genetic Programming of Liver and Pancreas Progenitors: Lessons for Stem-Cell Differentiation.” *Nature Reviews Genetics*.

Zhou, Qiao et al. 2007. “A Multipotent Progenitor Domain Guides Pancreatic Organogenesis.” *Developmental Cell* 13(1): 103–14.

Zorn, Aaron M, and James M Wells. 2009. “Vertebrate Endoderm Development and Organ Formation.” *Annual review of cell and developmental biology* 25: 221–51.

## 7 Publications

**Scheibner K.***, Burtscher I.*, Schirge S.*, Yang D., Sterr M., Yang D., Irmler M., Beckers J., Cernilogar F., Schotta G., Lickert H. **Definitive endoderm is formed by epithelial-to-epithelial transition during gastrulation.** Nature, 2020, submitted

Mahaddalkar P.U. *, **Scheibner K.***, Pfluger S., Ansarullah, Sterr M., Beckenbauer J., Irmler M., Beckers J., Knöbel S, Lickert H. **Generation of pancreatic b-cells from CD177$^{+}$ anterior definitive endoderm.** Nature Biotechnology. 2020, doi: 10.1038/s41587-020-0492-5 (*Co-first author).

Bakhti, M. $^{+,}$ *, **Scheibner K.***, Tritschler S, Bastidas-Ponce A., Tarquis-Medina M., Theis F.J., Lickert H$^{+}$. **Establishment of a high-resolution 3D modeling system for studying pancreatic epithelial cell biology in vitro**. Molecular Metabolism. 2019 Sep 12; https://doi.org/10.1016/j.molmet.2019.09.005 (*Co-first author; $^{+}$Co-corresponding).

Bastidas-Ponce A*, Tritschler S*, Dony L, **Scheibner K**, Tarquis-Medina M, Salinno C, Schirge S, Burtscher I, Böttcher A, Theis FJ+, Lickert H$^{+}$, Bakhti M$^{+}$. **Comprehensive single cell mRNA profiling reveals a detailed roadmap for pancreatic endocrinogenesis.** Development. 2019 Jun 17;146(12) (*Co-first author; $^{+}$Co-corresponding).

**Scheibner, K**.*; Bakhti, M. *; Bastidas-Ponce, A.; Lickert, H. **Wnt signaling: Implications in endoderm development and pancreas organogenesis.**
Curr. Opin. Cell Biol. 2019 61, 48-55 (*First co-authors).

Cernilogar, F.M ; Hasenöder, S.$^{\#}$ ; Wang, Z.$^{\#}$ ; **Scheibner, K.**$^{\#}$ ; Burtscher, I. ; Sterr, M. ; Smialowski, P. ; Groh, S. ; Evenroed, I.M. ; Gilfillan, G.D. ; Lickert, H.° ; Schotta, G. **Pre-marked chromatin and transcription factor co-binding shape the pioneering activity of Foxa2**. Nucleic Acids Res., (2019) ($^{\#}$Second co-authors).

Bastidas-Ponce A*, **Scheibner K***, Lickert H, Bakhti M. **Cellular and molecular mechanisms coordinating pancreas development.** Development. 2017 Aug 15;144(16):2873-2888. Review. (*First co-authors).

## 8 Abbreviations

| | |
|---|---|
| °C | Centigrade |
| µm | micrometer |
| Act A | Activin A |
| aPKC | Atypical protein kinase C |
| Arx | Aristaless related homeobox |
| BMP | Bone morphogenetic proteins |
| BSA | Bovine serum albumin |
| CD | Cluster of differentiation |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| ELISA | Enzyme-Linked ImmunoSorbent Assay |
| EP | Endocrine precursors |
| EtOH | ethyl alcohol |
| ε-cells | Epsilon-cells |
| FACS | Fluorescence Activated Cell Sorting |
| F-actin | Filamentous actin |
| FGF | Fibroblast growth factors |
| Fzd4 | Frizzled Class Receptor 4 |
| GATA4 | GATA Binding Protein 4 |
| GFP | Green fluorescent protein |
| Glis3 | GLIS Family Zinc Finger 3 |
| GS inh XX | γ-Secretase Inhibitor XX |
| GSIS | Glucose stimulated insulin secretion |
| Hes1 | Hairy and enhancer of split-1 |
| HHEX | Hematopoietically-expressed homeobox protein |
| Hnf1β | Hepatocyte nuclear factor 1β |
| hPECs | Human pancreatic epithelial cysts |
| hrs | hours |
| Ins | Insulin |
| Insm1 | Insulinoma-associated protein 1 |
| Irx1/2 | Iroquois-class homeodomain protein |
| KCl | Potassium chloride |
| kg | kilogram |
| KRPH | Krebs Ringer phosphate HEPES |
| M | Molar |
| MACS | Magnetic Activated Cell Sorting |
| MafA,B | MAF BZIP Transcription Factor A/B |
| min | Minutes |
| mL | Millilitre |
| mM | Millimolar |
| NA | Not assessed |
| NeuroD1 | Neuronal Differentiation 1 |
| ng | Nanogram |
| Nkx2.2 | Homeobox protein Nkx-2.2 |
| Nkx6.1 | Homeobox protein Nkx-6.1 |

| | |
|---|---|
| Pax4, 6 | Paired box protein Pax-4 and 6 |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PFA | Paraformaldehyde |
| PGT | Primitive gut tube |
| PP1, 2 | Pancreatic progenitor 1, 2 |
| qPCR | Quantitative PCR |
| Rfx6 | Regulatory factor X 6 |
| RNA | Ribonucleic acid |
| ROCK | Rho-associated protein kinase |
| rpm | Revolution per minute |
| RT | Room temperature |
| SIX1,2 | SIX homeobox 1,2 |
| Sox9 | SRY-Box 9 |
| T3 | 3,5,3'-triiodothyronine |
| TFs | Transcription factors |
| TGFβ | Transforming growth factor beta |
| TPB | (2S,5S)-(E,E)-8-(5-(4-(Trifluoromethyl)phenyl)-2,4-pentadienoylamino)benzolactam |
| Tris | Tris(hydroxymethyl)aminomethane |
| Ucn3 | Urocortin 3 |
| VitC | Vitamin C |
| VitE | Vitamin E |
| Wnt | Wingless signaling |
| YAP1 | yes-associated protein 1 |
| α-cells | Alpha-cells |
| β-cells | Beta-cells |
| δ-cells | Delta-cells |
| μL | Microliter |

# 9 Acknowledgements

'*I'm so clever, that sometimes I don't understand a single word of what I'm saying*.'
– Oscar Wilde

Firstly, I would like to express my sincere gratitude to my family. My **parents** for believing in me and giving me the freedom and trust to take my own decisions resulting in who I am and where I am now. For their unconditional support and love throughout my life. My **sister** for always being my companion during good and bad times, for loving and protecting me. My **grandparents**, for their support and believe in me and my work.

To the two pillars of my life, **Steffi** and **Josi**, who make my life the most beautiful journey to be in. Both fighting, protecting, loving and strengthening me every day. Believing in me, supporting me and encouraging me to achieve whatever I aim for.

I would like to pay my special regards to **Heiko** for offering me a PhD project in this special lab. For all the trust, respect and support of my work. For guiding and encouraging me, even when the road got tough. For creating a lab of scientific freedom and joy; a base for friendships and success. To **Ingo** and **Silvi** for accompanying me on this trip right from the start and for their continuous support throughout this long journey. To **Mosi** for opening the doors to join his group, for the valuable experience to work in a great team and for your unlimited support and appreciation. All of you for teaching me patience, stamina, independency, for letting me grow as a scientist.

I am incredibly grateful for my chicas **Aimée**, **Marta**, **Perla**, my bello **Ciro** and my little indian princess **Pallavi**. For our lovely lunch times, spicing up lab work and bringing more color into my lab life but especially also outside of the lab. For making hard times easier, for making me laugh until I cry and for partying with me until sun rise. To our lovely **Donna** for holding us together, for helping, listening and celebrating with us. To **Anna**, **Rosi** and **Benoite** for helping and cheering me up. To **Jessi**, **Julia B.**, **Bianca** and **Anne** for your technical support and the laughs we had together.
To all my family, friends, colleagues of **IDR** and my **thesis committee, Christina Scheel and Thomas Floss,** thank you for guiding, helping and accompanying me on this long journey.