Wnt signaling: implications in endoderm development and pancreas organogenesis

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Short Title: Wnt signaling in endoderm and pancreas development

Abstract

The pancreas is derived from the foregut endoderm during embryonic development. After gastrulation and endoderm germ layer formation complex morphogenetic events coupled with cell differentiation programs pattern the gut tube and induce pancreas organogenesis. This results in formation of exocrine, ductal and hormone-producing endocrine cells responsible for blood glucose homeostasis. The malfunction of the endocrine pancreas leads to diabetes mellitus, which cannot be stopped or reversed by the current standard treatments. Thus, intense efforts to regenerate or replace the lost or dysfunctional insulin-producing β-cells are on the way. This depends on identifying the factors that coordinate pancreas organogenesis. Here, we highlight the contribution of canonical and non-canonical Wnt signaling branches in orchestrating endoderm formation, pancreatic morphogenesis as well as endocrine cell formation and function.

Key words: Endoderm; gut tube; pancreas; β-cell; canonical Wnt/β-catenin; non-canonical Wnt/PCP; diabetes; islet.

Introduction

During gastrulation the three germ layers mesoderm, ectoderm and endoderm are formed and the body axes are established. Subsequent patterning of endoderm leads to the formation of the fore-, mid- and hindgut region. The primitive gut tube gives rise to various organs along the anterior-posterior axis, including the thymus, thyroid, lung, liver, pancreas and gastro-intestinal tract. Among these, pancreas anlage specification initiates by appearance of two buds consisting of multipotent pancreatic progenitors (MPCs) from a patterned and specified region of the foregut endoderm. The expansion and fusion of these buds generate a single organ that consequently gets patterned into central trunk and peripheral tip domains. Further differentiation of tip and trunk cells forms unipotent acinar and bipotent duct/endocrine progenitors, respectively [1,2]. A combination of signaling events and neighboring tissue interactions coordinate growth, patterning and differentiation of pancreatic progenitors that undergo consecutive lineage restrictions towards acinar, ductal and endocrine cells. In the adult pancreas, endocrine cells are clustered into the islets of Langerhans that secret different hormones, such as insulin and glucagon to regulate glucose homeostasis and energy metabolism [3].

A variety of signaling pathways coordinate different stages of endoderm development and pancreas organogenesis [4]. Among these, Wnt signaling is an evolutionary conserved pathway with two distinct branches: the canonical and non-canonical branch[5]. Both pathways are activated by binding of different Wnt ligands to the corresponding Frizzled (Fzd) receptors and their co-receptors. In canonical Wnt/β-catenin signaling, the Wnt-Fzd interaction activates Disheveled (Dvl) to prevent β-catenin phosphorylation, ubiquitination and degradation by a protein degradation complex consisting of Axin, CK-1, APC and GSK3β [6]. As the result, β-catenin translocates into the nucleus and activates Tcf/Lef transcription factor (TF) for the activation of canonical Wnt/β-catenin target genes (Figure 1a) [5,7]. In contrast, Wnt/planar cell polarity (PCP) signaling is β-catenin-independent and coordinates cell polarity and tissue architecture through secondary effectors, such as small GTPases and actin cytoskeleton (Figure 1b) [8,9]. Despite distinct functions, multiple functional crosstalk exists between the two Wnt signaling branches [10,11]. In this review, we first discuss current understanding of Wnt signaling function during endoderm development. Because excellent reviews on Wnt signaling in several endoderm-derived organs have already been published elsewhere [12–15], we focus on the role of this signaling pathway during pancreas organogenesis. We highlight recent findings on how Wnt pathways regulate pancreatic induction, lineage segregation as well as endocrine cell maturation and function.

Endoderm formation during mouse gastrulation

As gastrulation starts, pluripotent epiblast cells undergo an epithelial-to-mesenchymal transition (EMT) and delaminate from the epiblast into the PS between the epiblast and visceral endoderm (VE) layer to give rise to mesoderm or endoderm. Definitive endoderm (DE) cells then intercalate into the overlying VE forming one epithelial layer (Figure 2a) [16]. Based on transplantation and fate mapping experiments together with studies with mouse embryonic stem cells, it is believed that endoderm derives from bipotent mesendoderm progenitors in the anterior PS (APS) [17–19] (Figure 2a). We and others have noticed that a small population of Foxa2^{+} endoderm progenitors with an epithelial morphology is located distal to the APS, which might directly delaminate into the VE without a classical EMT [20,21]. These observations raise the question if an APS Foxa2^{+} epiblast progenitor gives rise to the entire endoderm. This would be against the common assumption that the endoderm is derived from mesendoderm progenitors [17–19]. Further analysis will reveal, if endoderm derives from a bipotent progenitor population and if these cells undergo EMT or rather a process in which endoderm progenitors keep their epithelial characteristics.

Gastrulation requires a tightly regulated spatio-temporal signaling network, which is orchestrated by so called gastrula organizer tissues, to induce and pattern the three germ layers. The onset of gastrulation is controlled by a positive signaling feedback loop involving bone morphogenetic protein (BMP), Wnt/β-catenin and Nodal signaling at the posterior site of the embryo. At embryonic day (E) 6.0 the distal VE (DVE) migrates to the prospective anterior site of the embryo forming the AVE, which secretes Nodal and Wnt signaling inhibitors including left-right determination factor 1 (Lefty1), Cerberus 1 (Cer1) and Dickkopf 1 (Dkk1) blocking Nodal and Wnt signaling on the anterior side of the embryo. Meanwhile, the posterior epiblast, far away from the AVE signals, receives Nodal and Wnt/β-catenin signaling leading to the initiation of PS formation [5].

By the end of gastrulation, the endodermal sheet is patterned into anterior and posterior DE fates giving rise to fore-, mid- and hindgut [16,22]. Even though much is known about signaling pathways inducing germ layer formation, the precise regulation of signals patterning gut endoderm towards an anterior vs posterior fate is not fully explained. Loss-of-function experiments of the Wnt3a ligand and its downstream effectors, including Lef1/Tcf1 double knockouts and β-catenin mutants, have demonstrated that canonical Wnt signaling is required to initiate PS and consequently the formation of endoderm and mesoderm [23–25]. Furthermore, the removal of β-catenin in Cytokeratin19-positive mesendoderm progenitors results in ectopic cardiac mesoderm formation at the expense of endoderm, highlighting the relevance of Wnt/β-catenin signaling in endoderm induction [26]. Indeed, the Wnt/β-catenin signaling pathway directly regulates the expression of SRY HMG-box transcription factor 17 (Sox17) in DE. Sox17 lineage tracing of DE cells showed the dependency of mid- and hindgut, but not foregut formation on Wnt/β-catenin signaling

[27]. These studies show that Wnt/β-catenin signaling is fundamental for endoderm induction and posterior endoderm patterning (Figure 2b).

Human endoderm formation *in vitro*

Understanding the signaling requirement to differentiate human embryonic stem cells (hESCs) to endoderm, pancreatic progenitors and its derivatives, such as insulin-producing β-cells, is crucial for cell-replacement therapies. In the last years, great progress has been made in generating human stem cell (SC)-derived β-like cells (SC-β) *in vitro*. However, SC-β cells are still immature and non-functional due to the remarkable heterogeneity during every step of *in vitro* differentiations. The current protocols induce DE by activation of WNT/β-catenin and Nodal signaling resulting in a seemingly homogenous DE population characterized by expression of pan-endoderm markers CXCR4$^+$/c-Kit$^+$ or FOXA2$^+$/SOX17$^+$. Already in the PS, anterior *vs* posterior endoderm fate is patterned by morphogen gradients of Nodal, Wnt and Bmp signaling factors [22,28,29]. Following gastrulation, distinct subpopulations of ADE, that give rise to liver and pancreas, have been observed in the endodermal layer before upregulation of organ-specific genes [30]. Accordingly, endoderm specification and patterning *in vivo* requires highly fine-tuned signaling and it is questionable if this can be mimicked *in vitro*.

In an attempt to dissect endoderm heterogeneity *in vitro*, we screened a library of 330 monoclonal surface antibodies and discovered several DE subpopulations discriminated by expression of different cell surface epitopes. Among these DE subpopulations, we identified CD177$^+$ and CD275$^+$ ADE subpopulations that are already specified towards the pancreas or liver fate, respectively. These two populations receive differential WNT signaling, where Wnt/PCP pathway is activated in CD177$^+$ population and canonical Wnt signaling in CD275$^+$ cells. These findings indicate that also *in vitro* the endoderm is patterned by the interaction with neighbouring cells, which is translated into the generation of different organ progenitors (unpublished).

Recent work suggests that hESCs require a pre-exposure with WNT/β-catenin signaling to obtain the competence responding to Nodal signaling [31]. Similarly, *in vivo* epiblast cells are first exposed to Wnt/β-catenin and then Nodal signaling, when allocating to mesoderm and endoderm. These findings demonstrate that a short wave of Wnt/β-catenin signaling is necessary to allow Nodal signaling to generate pan-endoderm. Our current understanding of signaling pathways regulating differentiation of stem cells into the desired fates, such as ADE *vs* PDE is incomplete. This is reflected by the various approaches to induce endoderm used by prominent hESC differentiation protocols, which will probably generate divergent flavors of endoderm biased towards distinct organ fates [32–34]. Thus, a more homogeneous initiation of the desired organ fate will not only improve differentiation efficiency, but likely also the maturation and functionality of terminally differentiated cells.

Wnt signaling controls pancreatic specification and patterning

Several signaling cascades including Wnt/β-catenin regulate different stages of early pancreas development. The formation of foregut endoderm as well as induction and specification of pancreas and liver rely on the repression of the Wnt/β-catenin pathway (Figure 3) [35]. Consistently, forced expression of β-catenin from onset of pancreas induction leads to pancreas agenesis. In mouse, this is partially through increased Hedgehog signaling, which represses pancreatic induction [36]. In *Xenopus*, Wnt/β-catenin repression triggers expression of the homeobox TF *Hhex* in the foregut endoderm for pancreatic and liver induction [35]. Later, the non-canonical function of Wnt5a in ventral foregut endoderm favors pancreatic over hepatic fate in this animal model [37]. In contrast, Wnt2 and Wnt2bb function is essential for liver induction and growth in zebrafish [38], highlighting the tight temporal modulation of Wnt signaling for liver formation (Figure 3). Recently, Fzd4 as the receptor for Wnt5a has been shown to regulate pancreas development. Treatment of *Xenopus* pancreatic explants by retinoic acid induces *Fzd4* expression to trigger pancreatic progenitor formation and differentiation [39]. Overall, these studies highlight that the precise spatio-temporal regulation of Wnt signaling branches is required for the induction of pancreatic fate.

After induction, efficient pancreas expansion relies on Wnt/β-catenin activity, through maintenance and proliferation of pancreatic progenitors (Figure 3) [40]. Deletion of Wnt/β-catenin in early pancreatic progenitors reduces the MPC pool that subsequently decreases the number of both exocrine and endocrine cells [41]. Moreover, Wnt signaling regulates pancreatic epithelial morphogenesis and patterning. It has been shown that together with the scaffolding protein Afadin, the non-canonical downstream effector RhoA controls lumen formation and epithelial remodeling [42]. On the other hand, Wnt/β-catenin coordinates tip-trunk patterning by inhibiting Notch signaling in tip cells to prevent their conversion towards the trunk fate (Figure 3) [43]. However, whether the cell-cell adhesion function of β-catenin contributes to this patterning remains unknown. A recent study has shown that the differential expression of the cell-cell adhesion protein p120-catenin (p120ctn) coordinates tip-trunk patterning and directs cell-fate decision [44,45]. The upstream cues regulating this process have not been identified and it will be interesting to investigate whether modulation of Wnt signaling plays a role. Additionally, the decision between ductal and endocrine fate is regulated by mechanotransduction dictated by extracellular matrix and integrin signaling [46]. Due to the crosstalk between cell-cell adhesion, mechanotransduction, Hippo and Wnt signaling, future work should address how morphogenesis and cell-fate decision are orchestrated during pancreas development.

Wnt signaling coordinates endocrine cell differentiation and morphogenesis

During endocrinogenesis, bipotent cells within the pancreatic epithelium give rise to endocrine progenitors expressing Neurogenin3 (Neurog3) that further differentiate into hormone-producing cells. The

delamination of endocrine cells from the epithelium and subsequent clustering forms islets of Langerhans [47,48]. Several studies have provided evidence that the repression of Wnt/β-catenin signaling is essential for endocrinogenesis (Figure 3). This is supported by the finding that the deletion of Wnt9a and subsequent reduced levels of Tcf7l2 induces endocrine genes, such as Nkx2-2 and Pax4 [49]. The crosstalk between pancreatic epithelium and the surrounding mesenchyme is important for modulating the Wnt pathway for endocrine differentiation. This occurs by producing and secreting non-canonical Wnt5a by mesenchyme that in turn triggers the expression of Wnt inhibitors Sfrp3 and Dkk1 in endocrine progenitors for endocrine fate allocation (Figure 3) [50]. Because Wnt5a is also expressed in pancreatic epithelial cells, it is unclear how expression of this ligand is coordinated in epithelial and/or mesenchymal cells to regulate endocrine cell formation. More evidence for non-canonical Wnt/PCP pathway in endocrinogenesis comes from a study showing active PCP in pancreatic progenitors and that two PCP components Celsr2 and 3 are essential for endocrine cell differentiation (Figure 3) [51]. Future work should address whether Wnt/PCP is involved in regulating endocrine induction and subtype specification, a process that has not yet been fully understood. Non-canonical Wnt signaling is well-known to establish planar cell polarity and is essential for tissue morphogenesis and architecture. There is accumulating evidence that the polarity, morphogenesis and differentiation of endocrine cells are tightly interlinked [52,53]. For instance, EGFR signaling through PI3K and Rac1 modulates apico-basal polarity in endocrine progenitors to control endocrine cell differentiation and morphogenesis [53]. Furthermore, deletion of Cdc42 in pancreatic progenitors perturbs epithelial morphogenesis and endocrine cell formation [54]. Although both Rac1 and Cdc42 are downstream effectors of Wnt/PCP [55], it is unknown if this pathway mediates the coupling of endocrine cell morphogenesis and differentiation. Moreover, Cdc42 regulates endocrine cell delamination through modulation of actin cytoskeleton dynamics [56]. This is interesting as Wnt5a has also been shown to induce islet delamination and migration [57]. However, uncovering how the Wnt pathway coordinates delamination process and islet morphogenesis needs further attention.

Wnt signaling during β-cell maturation, function and failure

After differentiation, β-cells undergo a postnatal maturation process to acquire a glucose-responsive phenotype [58]. Evidence on the involvement of Wnt signaling during β-cell maturation comes from a study showing the existence of two distinct β-cell populations based on the expression of the Wnt/PCP downstream effector Flattop (Fltp) [59]. Cells lacking Fltp reporter activity represent a proliferative and more immature population, while Fltp reporter or lineage positive cells are metabolically active and mature β-cells (Figure 3) [60]. How Wnt/PCP signaling coordinates β-cell maturation is still under investigation. One possible scenario is that Wnt/PCP pathway orchestrates islet architecture, which in turn impacts β-cell

maturation and function [60,61]. In this regard, Wnt4 and Wnt5a increase the expression of β-cell maturation markers in 3D *in vitro* cultures (Figure 3) [60]. Furthermore, the function of mTOR signaling is critical for β-cells to acquire glucose-responsive capacity [62]. In intestine and liver, mTORC1 hampers Wnt/β-catenin signaling, by modulating membranous Fzd levels [63]. Whether such a crosstalk between mTOR and Wnt signaling regulates β-cell maturation requires further investigation. Moreover, the contribution of different Wnt branches to β-cell function deserves more studies. In this context, increased β-catenin levels regulate insulin secretion by modulating intracellular actin remodeling [64]. A recent study has indicated the involvement of GSK3β, the negative regulator of β-catenin, in β-cell function. In type 2 diabetic (T2D) islets, GSK3β impacts the function of PDX1 and impairs insulin secretion [65]. Notably, the capacity of β-cells to secret insulin is recovered by GSK3β pharmacological inhibition, highlighting the possible targeting of Wnt signaling-related genes for T2D treatment.

The dysregulation of Wnt pathway contributes to the development of metabolic disorders such as diabetes mellitus. Genome-wide association studies have identified several Wnt signaling-related genes for susceptibility to develop T2D. Some of these genes encode proteins implicated in islet formation and function. The prime example is Tcf7l2/Tcf4 that regulates β-cell growth and insulin secretion [66–68]. Furthermore, TCF7L2 mediates β-cell proliferation induced by glucagon-like peptide-1 (GLP-1) [69]. Importantly, the function of Tcf7l2 in islets is not restricted only to β-cells. In this regard, specific deletion of Tcf7l2 in α-cells reduces glucagon secretion and α-cell mass [70]. Because *TCF7L2* is one of the most relevant T2D GWAS gene, a deeper understanding of the tissue-specific functions of this TF in metabolic regulation is further required. Future studies not only should identify the developmental impact of *TCF7L2* mutations on islet homeostasis but also define to which extent *TCF7L2* mutations in different organs, such as liver, gut and pancreas contribute to diabetes development.

Conclusion

A comprehensive understanding of endoderm development and pancreas organogenesis allows *in vitro* differentiation of stem cells into islet-like clusters (ILCs). Deciphering the spatio-temporal function of developmental signaling and processes regulating endoderm and pancreas development does not only allow to isolate proper endodermal progenitors and their efficient priming towards the pancreatic fate, but also enables differentiation of mature and functional ILCs. Among these, Wnt/β-catenin pathway regulates cell expansion and differentiation, whereas Wnt/PCP controls cell dynamics, morphogenesis and differentiation to orchestrate tissue and organ architecture required for function. Future work should address how a switch from canonical to non-canonical Wnt pathways or *vice versa* regulates pancreatic lineage formation and establishes pancreatic shape and architecture that will help to generate stem cell-derived functional ILCs and guide tissue engineering approaches, respectively. Additionally, *ex vivo* modeling systems such as 3D

organoids will help to explore the function of Wnt signaling during human pancreas organogenesis. Finally, targeting Wnt-related genes might identify triggering routes of endocrine cell regeneration. Further studies need to show whether modulating Wnt/β-catenin and/or Wnt/PCP enables β-cell neogenesis, proliferation and redifferentiation. This together with recent advancements in targeted delivery of small molecules will hopefully aid to construct novel regenerative approaches for diabetes therapy.

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Conflict of Interest

The authors declare no conflict of interest

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Annotated references

31. This study shows that hESCs require to be pre-exposed to Wnt signaling, to be able to respond to ACTIVIN morphogen activity. Special interest (•)

42. One of the few study that show the molecular regulators of pancreatic epithelial formation and tubulogenesis. The authors show that cooperative function of the scaffolding protein Afadin and RhoA regulates pancreatic epithelial remodeling and lumen formation. Special interest (•)

44. This study shows that niche factors dictate the lineage decision during pancreas development. The authors show that differential expression of p120-catenin (p120ctn) regulates the patterning of tip and trunk domain as well as endocrine vs ductal fate. Outstanding interest (••)

46. This study shows the ductal and endocrine fate decision through mechanosignaling axis. Such signaling is regulated through interaction of integrin α5 to ECM components indicating pancreatic lineage decision by surrounding niche factors. Outstanding interest (••)

52. This study shows the mechanistic detail of endocrine cell delamination through regulation of actomyosin contractility. Special interest (•)

53. This study shows the regulation of endocrinogenesis by cell polarity. EGFR signaling through PI3K and Rac1 reduces the apical domain size of endocrine progenitors that consequently inhibits Notch signaling and increases expression levels of Ngn3. Outstanding interest (••)

65. High-sensitivity mass spectrometry-based proteomics disclose a regulatory loop between GSK3β and PDX1 dysregulated during pathogenic conditions that impair insulin secretion in mouse and human. Special interest (•)

70. This study shows a cell-autonomous function of Tcf7l2, one of the most GWAS-gene related to T2D predisposition. Specific deletion of Tcf7l2 in alpha-cells results in reduction in alpha-cell mass, Gcg and MafB expression and Gcg secretion. Special interest (•)

Figure legends

Figure 1: Canonical and non-canonical signaling pathway

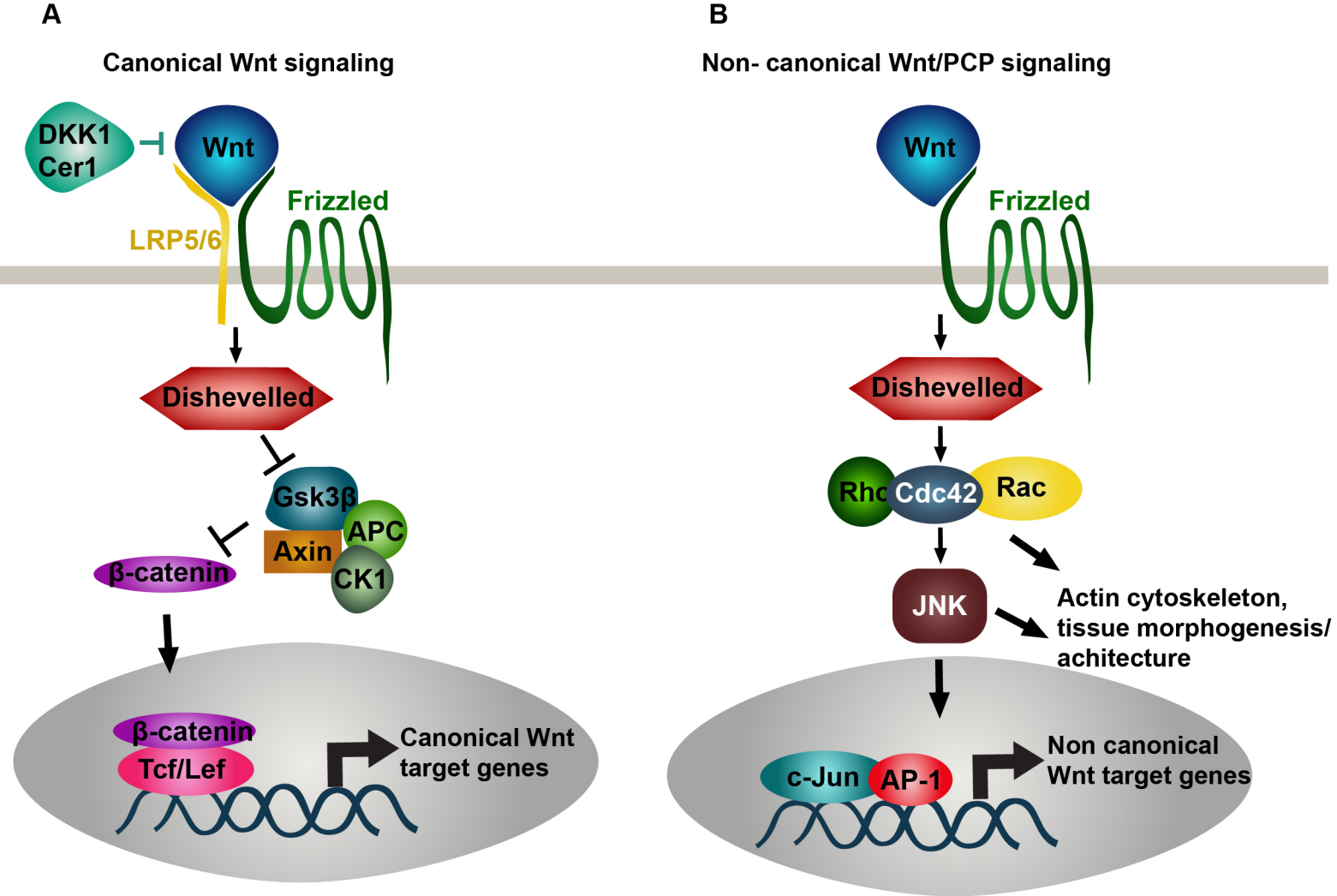
Scheme of canonical Wnt/β-catenin and non-canonical Wnt/PCP signaling pathway is shown. **(a)** Upon Wnt ligand-receptor/co-receptor interaction, Disheveled is activated, which in turn inactivates the β-catenin destruction complex (Axin, CK1, APC, GSK3β). β-catenin is then translocated to the nucleus, activates Tcf/Lef TFs to induce transcription of Wnt/β-catenin target genes. **(b)** The Wnt/PCP pathway is activated by binding of Wnt ligands to its receptor leading to the activation of Disheveled, small GTPases and JNK resulting in either activation of non-canonical Wnt target gene expression or cytoskeletal rearrangements.

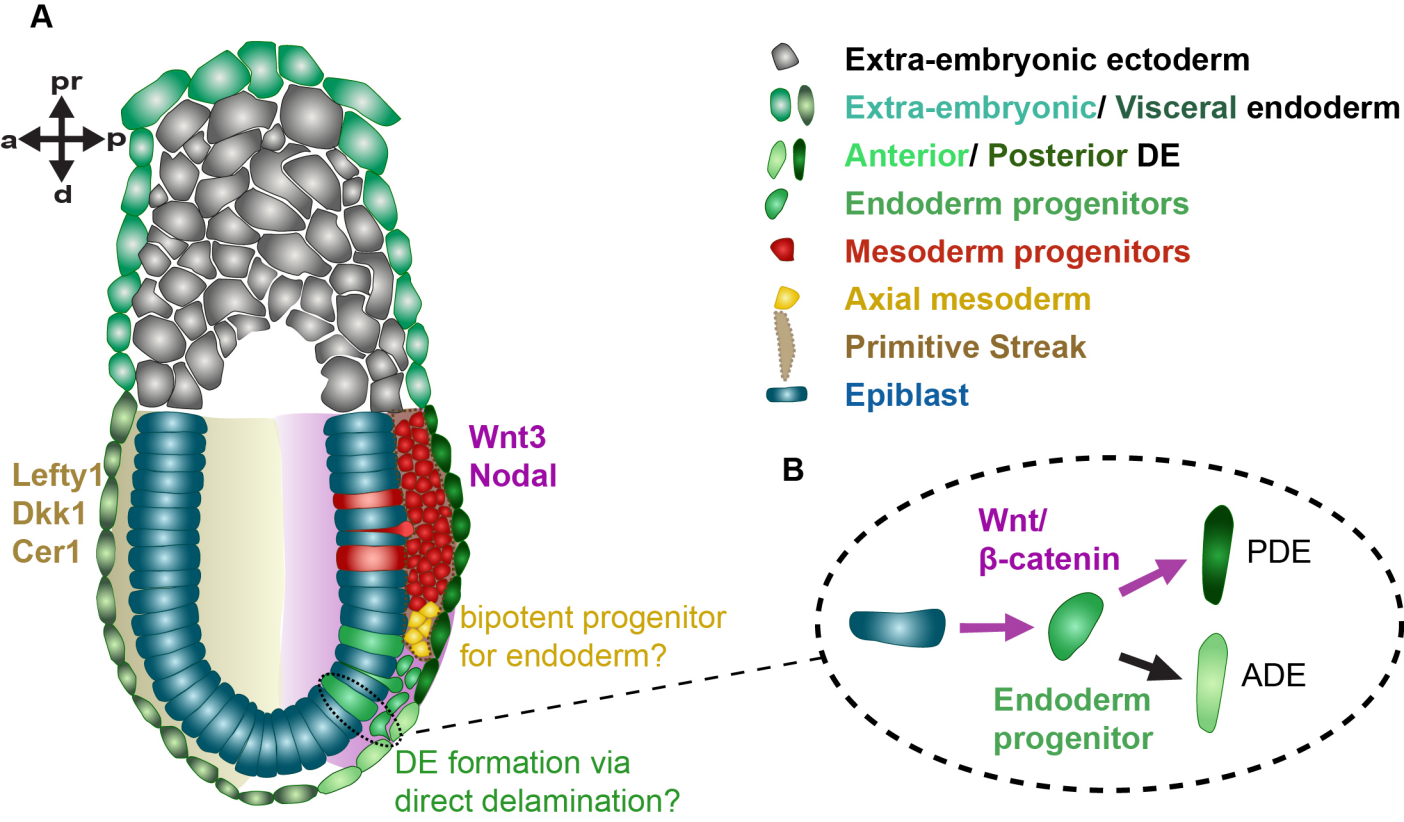
Figure 2: Endoderm formation during gastrulation

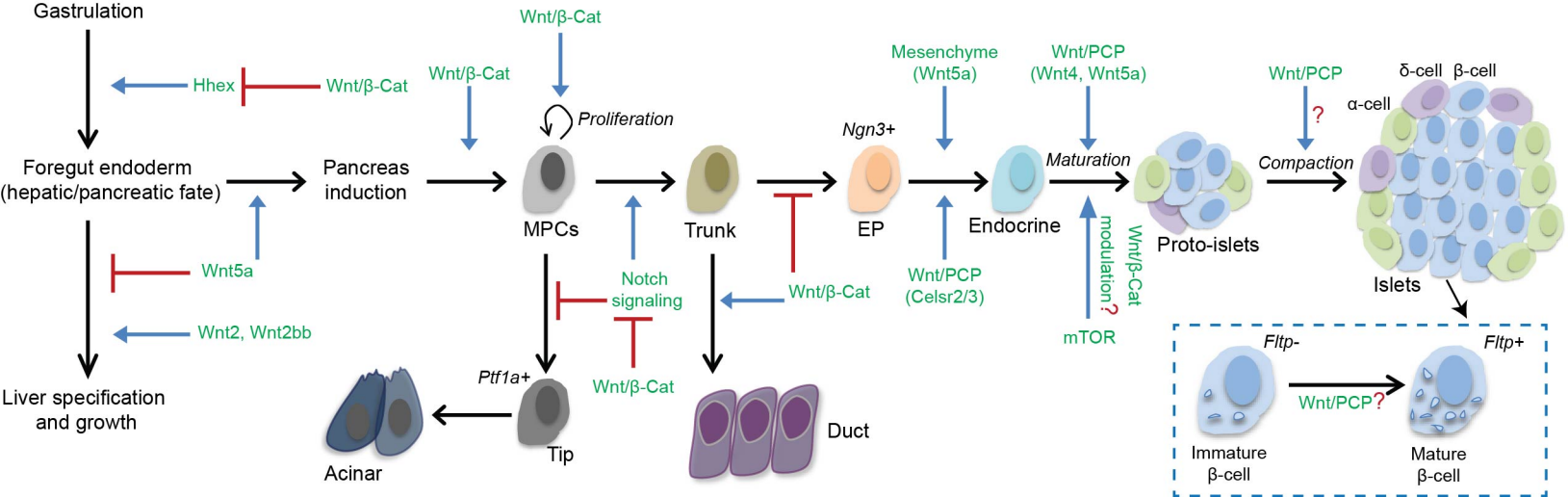
Schematic representation of endoderm formation during mouse gastrulation. **(a)** Wnt3a and Nodal signaling at the posterior side of the embryo induces the formation of mesoderm (red) and endoderm (green). Endoderm located distal to the anterior primitive streak (APS) might not derive from a bipotent progenitor (yellow) and directly delaminates into the VE without undergoing a classical EMT process (dashed black circle). **(b)** Specification of pluripotent epiblast cell towards DE. Wnt/β-catenin signaling is required for DE induction and PDE patterning, but not for ADE formation.

Figure 3: Wnt signaling regulates multiple steps of pancreas organogenesis

Schematic representation shows the precise spatio-temporal regulation of canonical and non-cononical Wnt signaling at different steps of mouse pancreatic induction, expansion, patterning and differentiation. Compared to the intense studies on the Wnt signaling function during early pancreas development, much remains to be discovered for the interplay of the function of Wnt/β-catenin and Wnt/PCP branches during establishment of pancreas architecture, lineage segregation as well as terminal differentiation and maturation of endocrine cells. Blue arrows indicate activation and red lines indicate inhibition. MPCs, multipotent pancreatic progenitors; EP, endocrine progenitors/precursors. ? indicates the requirement of more experimental evidence.


A
Canonical Wnt signaling
DKK1
Cer1
Wnt
Frizzled
LRP5/6
Dishevelled
Gsk3β
APC
Axin
CK1
β-catenin
β-catenin
Tcf/Lef
Canonical Wnt
target genes
B
Non- canonical Wnt/PCP signaling
Wnt
Frizzled
Dishevelled
Rho
Cdc42
Rac
JNK
Actin cytoskeleton,
tissue morphogenesis/
achitecture
c-Jun
AP-1
Non canonical
Wnt target genes


A
pr
a
p
d
Lefty1
Dkk1
Cer1
Wnt3
Nodal
bipotent progenitor for endoderm?
DE formation via direct delamination?
Extra-embryonic ectoderm
Extra-embryonic/ Visceral endoderm
Anterior/ Posterior DE
Endoderm progenitors
Mesoderm progenitors
Axial mesoderm
Primitive Streak
Epiblast
B
Wnt/
β-catenin
PDE
ADE
Endoderm
progenitor


Gastrulation
Hhex
Wnt/β-Cat
Foregut endoderm
(hepatic/pancreatic fate)
Pancreas
induction
Wnt5a
Wnt2, Wnt2bb
Liver specification
and growth
Wnt/β-Cat
Wnt/β-Cat
Proliferation
MPCs
Notch
signaling
Wnt/β-Cat
Trunk
Wnt/β-Cat
Ptf1a+
Tip
Acinar
Duct
Ngn3+
EP
Mesenchyme
(Wnt5a)
Wnt/PCP
(Celsr2/3)
Endocrine
Wnt/PCP
(Wnt4, Wnt5a)
Maturation
modulation?
mTOR
Wnt/β-Cat
Proto-islets
Wnt/PCP
?
Compaction
δ-cell
β-cell
α-cell
Islets
Fltp-
Fltp+
Wnt/PCP?
Immature
β-cell
Mature
β-cell