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Wnt7b is required for epithelial progenitor growth and operates during epithelial-to-mesenchymal signaling in pancreatic development

Q1 Solomon Afelik*, Brandon Pool, Martin Schmerr, Christopher Penton, Jan Jensen*

Q2 *Department of Stem Cell Biology and Regenerative Medicine, Lerner Research Institute, Cleveland Clinic, NE3-251, 9500 Euclid Avenue, Cleveland, OH 44195, United States*

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ABSTRACT

Wnt signaling is a well conserved pathway critical for growth, patterning and differentiation of multiple tissues and organs. Previous studies on Wnt signaling in the pancreas have been based predominantly on downstream pathway effector genes such as β-catenin. We here provide evidence that the canonical-pathway member Wnt7b is a physiological regulator of pancreatic progenitor cell growth. Genetic deletion of *Wnt7b* in the developing pancreas leads to pancreatic hypoplasia due to reduced proliferation of pancreatic progenitor cells during the phase of pancreas development marked by rapid progenitor cell growth. While the differentiation potential of pancreatic progenitor cells is unaffected by *Wnt7b* deletion, through a gain-of-function analysis, we find that early pancreatic progenitor cells are highly sensitive to Wnt7b expression, but later lose such competence. By modulating the level and the temporal windows of Wnt7b expression we demonstrate a significant impact on organ growth and morphogenesis particularly during the early branching stages of the organ, which negatively affects generation of the pro-endocrine ($Ngn3^+$/$Nkx6.1^+$), and pro-acinar ($Ptf1A^+$) fields. Consequently, Wnt7b gain-of-function results in failed morphogenesis and almost complete abrogation of the differentiation of endocrine and acinar cells, leading to cystic epithelial metaplasia expressing ductal markers including Sox9, Hnf6 and Hnf1β. While Wnt7b is expressed exclusively in the developing pancreatic epithelium, adjacent mesenchymal cells in the organ display a direct trophic response to elevated Wnt7b and increase expression of Lef1, cFos and desmin. Of note, in contrast to the pancreatic epithelium, the pancreatic mesenchyme remains competent to respond to Wnt7b ligand, at later stages in development. We conclude that Wnt7b helps coordinate pancreatic development through autocrine, as well as paracrine mechanisms, and as such represents a novel bi-modal morphogen ligand.

Introduction

Tissue interactions between mesodermal cells and adjacent endodermal epithelial cells occur in development, homeostasis, and also during metaplastic growth of internal organs. During development, instructive inputs from paraxial gut-associated mesenchyme aid in endodermal organ formation and budding. During adult homeostasis and under regenerative processes, such relationships are often referred to as niche/progenitor signaling. In pancreatic cancer, recruitment of adjacent stromal tissues aids in growth and metaplastic maintenance of the tumor (Apte et al., 2013; Erkan et al., 2012; Feig et al., 2012; Hamada et al., 2013). For the most part, mesenchymal-to-epithelial signaling in the pancreas is mainly considered whereas the reciprocal signaling from epithelium-to-mesenchyme is less well understood.

In the case of pancreas, organ formation occurs when dorsal and ventral evaginations of the posterior foregut endoderm occur into in a dense layer of mesenchyme, creating nascent pancreatic buds. By embryonic day 11.5 these pancreatic epithelia initiates extensive branching morphogenesis whereby the initially stratified epithelium gradually transforms into a single-layered epithelium which grows and branches extensively into the mesenchymal cap. Epithelial growth outpaces the mesenchyme with an ensuing decrease in mesenchymal/epithelial ratio as development progresses. The importance of the mesenchyme for the growth of the embryonic pancreatic epithelium was first demonstrated in explants-type studies in which isolated pancreatic epithelium, stripped of the mesenchymal layer, underwent growth arrest, failure in exocrine cell differentiation, concomitant with accelerated differentiation of early-type glucagon-expressing endocrine cells. These effects could be rescued through recombination with

* Corresponding authors.
E-mail addresses: afeliks@ccf.org (S. Afelik), jensenj2@ccf.org (J. Jensen).

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mesenchyme of both pancreatic and non-pancreatic types (Golosow and Grobstein, 1962; Wessells and Cohen, 1967). Recent studies in which genetic tools were employed to achieve a more thorough and specific ablation of the mesenchyme *in vivo* revealed that the pancreatic mesenchyme is important for the growth of all pancreatic cell lineages (Landsman et al., 2011). Furthermore, genetic studies in the mouse have shown that Fgf10 emanating from the early pancreatic mesenchyme is required for the proliferation of the pancreatic epithelium (Bhushan et al., 2001; Hart et al., 2003; Miralles et al., 1998; Norgaard et al., 2003).

While the evidence for Fgf10 in mesenchymal-to-epithelial signaling is compelling, the level of Fgf10 expression dwindles to barely detectable levels by E13.5 in mice (Bhushan et al., 2001; Elghazi et al., 2002). This raises the possibility that Fgf10 might be important for the initial phase of pancreatic progenitor growth but not during the later growth phase of multipotent progenitors, in the period leading up to the secondary transition at which terminal differentiation initiates. In support of this notion, mouse pancreatic explant studies involving pharmacological inhibition of Fgfr signaling revealed that while Fgf signaling is required for the initial growth it is dispensable for later growth of epithelial explants (Greggio et al., 2013). Alternatively, during late stages of pancreas development, Fgf10 might operate in concert with other signaling pathways to promote pancreatic progenitor growth. Of note, recent studies have revealed the importance of canonical Wnt signaling in sustaining pancreatic progenitor using hydrogel-based, colony-forming assays (Greggio et al., 2013; Huch et al., 2013; Jin et al., 2013; Sugiyama et al., 2013).

Studies implicating a role of the Wnt pathway in pancreas development have largely been based on genetic perturbation of Wnt pathway components, rather than specific ligands. These studies reveal a cell-intrinsic requirement of the pathway in proliferation of epithelial progenitors. Pancreas-specific deletion of β-catenin leads to severely reduced pancreatic size and defects in acinar cell differentiation (Baumgartner et al., 2014; Dessimoz et al., 2005; Murtaugh et al., 2005; Wells et al., 2007). Conversely, overexpression of β-catenin in pancreas promote pancreatic epithelial growth (Heiser et al., 2006). Pancreatic overexpression of a dominant-negative Frizzled-8 receptor consisting of the Wnt ligand binding domain (Frz8CRD) results in reduced levels of unphosphorylated β-catenin in the pancreas and a drastically reduced size (Papadopoulou and Edlund, 2005). Deletion of *Pygopus2*, encoding a component of the β-catenin transcriptional complex, also leads to decreased pancreatic progenitor cell proliferation (Jonckheere et al., 2008). Of note, the importance of canonical Wnt signaling for pancreatic progenitor growth is not restricted to the epithelial compartment, as deletion of β-catenin specifically in the pancreatic mesenchyme also leads to reduced pancreatic size (Landsman et al., 2011).

The identity and source of the Wnt ligand responsible for activating the canonical Wnt pathway in either compartment have remained elusive. Here, we show that Wnt7b is exclusively expressed within the epithelial compartment and exerts effects on pancreatic epithelia and mesenchyme that is consistent with this ligand being responsible for the compartmental activation of canonical Wnt signaling. The expression of Wnt7b corresponds to the period when mesenchymal Fgf10 expression levels decrease. Pancreatic deletion of *Wnt7b* is sufficient to cause pancreatic hypoplasia to a degree similar to that observed in some cell intrinsic Wnt-pathway mutants in the developing pancreas (Jonckheere et al., 2008; Landsman et al., 2011). Interestingly, pancreatic gain-of-expression of Wnt7b results in abrogation of most cell differentiation; substantial expansion of pancreatic-associated mesenchyme; with an ensuing epithelial metaplasia resembling pre-cancerous lesions represented by polycystic ductal networks. These studies provide a basis for understanding Wnt7b-mediated organ growth, and uncover a novel epithelial-to-mesenchymal signaling pathway.

Materials and methods

Animals

The full length human *Wnt7b* cDNA clone was purchased from OriGene Technologies, Inc. This was cloned downstream of the tetracycline responsive promoter, in a modified version of the pTRE2 vector which contains an IRES-nEGFP cassette downstream of the multiple-cloning site. Doxycycline inducible expression of the construct was first validated by co-transfecting it with pCMV-rtTA into HEK293 cell. Nuclear EGFP expression was detected in the presence of doxycycline. A linearized fragment of the pTRE2-*Wnt7b*-IRES-nEGFP fragment was injected into fertilized one-cell embryos at the Case Western Reserve University transgenic and gene targeting facility. Out of a total of 30 animals recovered from injected embryos, 9 transgene positive founders (F0) were identified through PCR based genotyping, using DNA extracted from ear notches and primers specific for the transgene. Five of the founders transmitted the transgene, and following mating to Pdx1-tTA^{KI} we identified 3 independent lines which showed pancreas specific EGFP expression with a phenotype that is consistent among all 3 independent lines. To shut off transgene expression in Pdx1-tTA^{KI}; pTRE2-*Wnt7b*-IRES-nEGFP double transgenic embryos, doxycycline was injected intraperitoneally into pregnant females at a dose of 0.05 μg/g of body weight.

Wnt7b conditional null mice with *loxP* sites flanking exon3 of the *Wnt7b* gene were mated to Pdx1-Cre mice to induce pancreas specific *Wnt7b* null embryos. The creation of this mouse strain is previously described (Rajagopal et al., 2008). Generation of the Pdx1-Cre deleter mouse has been previously described (Gu et al., 2002). To determine Pdx1-Cre recombinase activity, Pdx1-Cre mice were mated to Rosa26$^{mT/mG}$ lineage tracer (Jackson Laboratory, stock number: 007576).

Immunofluorescence staining and microscopy

Immunofluorescence staining and analysis were performed as previously described (Norgaard et al., 2003) on $n \geq 3$ samples at all times points. Fresh tissue was dissected in cold 1X PBS and fixed in 4% paraformaldehyde (PFA) at 4 °C for 4 h or overnight. Fixed samples were frozen in OCT and 6 μm thick sections were prepared for histology. Tissue sections were washed in 1X PBS, blocked in 0.5% blocking reagent (PerkinElmer, Boston, MA) for 1 h and incubated with primary antibody overnight at room temperature. The following primary antibodies and dilutions were used: rabbit anti-amylase (1:100, Sigma: A82273); guinea pig anti-insulin (1:500, Dako: A0564); rabbit anti-somatostatin (1:500, Dako: A0566); mouse anti-glucagon (1:500, Sigma: G2654); Dolichos Biflorus Agglutinin (DBA) (1:100, Vector laboratories: FL-1031); rabbit anti-Sox9 (1:2000, Millipore: AB5535); goat anti-Pdx1 (1:2000, CV Wright, Nashville, TN, USA); mouse anti-Ngn3 (1:200, University of Iowa hybridoma bank: F25A1B3-c); mouse anti-Nkx6.1 (1:200, University of Iowa hybridoma bank: F64A6B4-c); rabbit anti-pHH3 (1:200, Upstate: 06-570); rabbit anti-Hnf1β (1:100, Santa Cruz: sc-22840); rabbit anti-Hnf6 (1:50, Santa Cruz: sc-13050); rabbit anti-PDGRRβ (1:100, Santa Cruz: sc-432); mouse anti-desmin (1:100, Santa Cruz; sc-271677); rabbit anti-β-catenin (1:100, NeoMarkers: RB-090-P1); rabbit anti-Ptf1a (1:2000, CV Wright, Nashville, TN, USA); mouse anti-acetylated tubulin (1:500, Sigma: T7451); rabbit anti-Active Caspase-3 (1:250, Promega: G748A); rabbit anti-scribbled (1:100, Santa Cruz: sc-28737); rabbit anti-cFos (1:100, Santa Cruz: sc-253); mouse anti-smooth muscle actin (1:100, Dako: 1A4); rabbit anti-Lef1 (1:100, Cell Signaling: #2230), with TSA™ signal amplification, TSA™ Tetramethylrhodamine System (PerkinElmer, Inc: NEL702001KT). Secondary antibodies were applied for 1 h at room temperature at 1:100 dilution (pre-absorbed secondary antibodies coupled to DyLight488, DyLight 594 or DyLight 649, Jackson Immunoresearch, West Grove, PA). For TSA™ signal amplification of Lef1, following primary antibody

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incubation, slides were incubated with biotinylated anti-rabbit antibody (undiluted, ZYMED: Cat#:95-6143B) for 30 min, washed and incubated with HRP–streptavidin (undiluted, ZYMED: Cat#:95-6543B) for 15 min. Slides were incubated with tetramethylrhodamine tyramide reagent (1:100, PerkinElmer, Inc: FP1014) for 15 min for signal development. Following stainings, slides were washed 3 × 5′ in 1X PBS and mounted in glycerol mount (20% glycerol in PBS). Images were acquired using IMAGEpro 4.1-7.0.

RNA in situ hybridization

Whole mount *in situ* hybridization was performed as previously described (Little et al., 2007) using DIG-labeled anti-sense *Wnt7b* mRNA probes. For signal generation, samples were incubated in NBT/BCIP overnight at 4 °C.

Morphometry, cell counting and assessment of organ size

For quantitative assessment of organ size pancreata from various genotypes, wild type littermates were isolated from E18.5 and E14.5 and weighed on a scale. To determine organ size at embryonic stages below E14.5 or analyze the relative proportion of various cell types, entire pancreatic tissue was sectioned and every fifth section was immunostained with the appropriate marker genes. The area of stained cells was then quantified with ImagePro Software (Media Cybermetics, Bethesda, MD). For quantification of relative proliferation rate the number of pHH3 (phosphorylated histone H3) positive nuclei per unit area was measured.

Results

Wnt7b is exclusively expressed by epithelial progenitors during pancreas development

Analysis of the expression of the Wnt-family members in the mid-gestational pancreas previously identified *Wnt11*, *Wnt2b*, *Wnt4*, *Wnt5a* and *Wnt7b* [(Heller et al., 2002), and data not shown]. Following transgenic overexpression of several of these Wnt ligands (Wnt1, Wnt2, Wnt4, Wnt5a, Wnt6 and Wnt7a), only Wnt1 and Wnt5a were shown to cause pancreatic hypoplasia. Using whole-mount *in situ* hybridization, we detected abundant *Wnt7b* transcripts in both dorsal and ventral pancreatic buds at E11.5 (Fig. 1A and E). It was previously suggested that Wnt7b is expressed in the pancreatic mesenchyme; (Heller et al., 2002), however using more improved staining techniques we have discovered that *Wnt7b* mRNA expression is limited to the pancreatic epithelium and absent from the pancreatic mesenchyme at all stages analyzed (E11.5–E15.5) (Fig. 1A–G'). *Wnt7b* expression remained restricted to the pancreatic epithelium as branching morphogenesis of the pancreatic epithelium becomes pronounced from E12.5 onwards (Fig. 1B, D, F, G, and G'). By E15.5 *Wnt7b* expression declined (Fig. 1D). The epithelial-restricted expression is consistent with a previous report of *Wnt7b* expression in the E13.5 pancreatic epithelium (Papadopoulou and Edlund, 2005). While low levels of *Wnt7b* transcripts are detectable within the definitive endoderm (Supplementary Fig. 1A), the highest levels of *Wnt7b* expression within the endoderm are restricted to the lung and pancreatic epithelium (Supplementary Fig. 1B).

Wnt7b is required for pancreatic organ growth prior to terminal cell differentiation

To define the role of *Wnt7b* during pancreas development, we utilized conditional *Wnt7b* mutant animals (*Wnt7b*$^{fl/fl}$) (Rajagopal et al., 2008). These mice allow for deletion of exon3 in the presence of Cre-recombinase, making it possible to generate tissue specific loss of *Wnt7b* function in the homozygous state. The Pdx1-Cre recombinase expressing mouse line (Supplementary Fig. 2) was crossbred to *Wnt7b* mutant mice to achieve pancreas-specific *Wnt7b* null embryos, hereafter referred to as *Wnt7b* PKO. Gross morphological examination of the distal foregut/midgut region of E18.5 embryos revealed a hypoplastic pancreas in *Wnt7b* PKO embryos (Fig. 2A). Quantitative assessment by weight shows no significant difference in body weight between WT, heterozygotes or *Wnt7b* PKO embryos (Fig. 2B) whereas the weight of E18.5 *Wnt7b* PKO pancreatic tissue is 57% that of wild type littermates (Fig. 2C). The ratio of pancreas to body weight also reveals a substantial reduction in pancreatic mass of *Wnt7b* PKO embryos relative to WT (Fig. 2D). Because the diminished pancreatic size could be due to late-gestational proliferative defects or cell death, we evaluated E14.5 *Wnt7b* PKO, heterozygotes and WT littermates. Similar to E18.5, these embryos were identical in size and weight, but the *Wnt7b* PKO pancreas was 42% of the wild type pancreatic weight (Supplementary Fig. 3).

The early impact on pancreatic growth could be explained by lack of formation of specific cell types where absence of such would cause a hypoplastic organ. We performed immunofluorescence staining to assess the composition of the major cell types in the pancreas by using antibodies directed to amylase for the acinar cell compartment, insulin, glucagon and somatostatin for the various cell types of the endocrine compartment and used the lectin DBA which specifically stains pancreatic ducts (Fig. 2E–H). Morphometric quantification revealed a similar proportion of the various cell types between *Wnt7b* PKO and WT pancreata at E18.5 (Fig. 2I–J). We next questioned if the reduced organ size could be accredited to a delay, or accelerated cell differentiation. Histological analysis of pancreatic tissue from E10.5 to E14.5 embryos with the differentiation markers indicated above showed that the onset and levels of expression of these markers were comparable between WT and *Wnt7b* PKO embryos, suggesting that the reduced pancreatic size in *Wnt7b* PKO embryos is not due to premature cell differentiation (data not shown).

Wnt7b is not a determinant of progenitor cell patterning

To further examine any potential effect of *Wnt7b* on the pancreatic progenitor cell state, we focused our analysis on pancreatic progenitor patterning. Prior to terminal cell fate differentiation, multipotent pancreatic progenitor cells (MPCs) become patterned into pro-acinar (TipPC) and pro-endocrine/duct compartments (TrPC) which are localized to the tip and trunk of the branched epithelium, respectively (Afelik et al., 2012; Schaffer et al., 2010; Zhou et al., 2007). The pancreatic progenitor markers Ptf1a, Nkx6.1 and Sox9 are initially expressed in all MPCs, but Ptf1a expression becomes restricted to the TipPC whereas the expression of Nkx6.1 and Sox9 becomes confined to the TrPC compartment. Histological analysis of *Wnt7b* PKO epithelium at E13.5 revealed no changes in the expression pattern and the relative proportion of the TrPC markers Nkx6.1 and Sox9 in *Wnt7b* PKO pancreatic epithelial cells (Fig. 3A–F). Likewise, a normal speckled pattern of Ngn3 expression was observed within the TrPC compartment (Fig. 3G, and H). The percentage of Ngn3-expressing epithelial cells in the *Wnt7b* PKO pancreas was comparable to WT (Fig. 3I). The unaltered expression and distribution of the lineage committing transcription factors are consistent with the normal differentiation pattern of endocrine and acinar cell fates observed at E18.5.

Wnt7b is required for pancreatic progenitor cell proliferation

The ultimate size of the pancreas is directly proportional to the mass of multipotent pancreatic progenitor cells present at the onset of pancreas development (Stanger et al., 2007). This prompted us to

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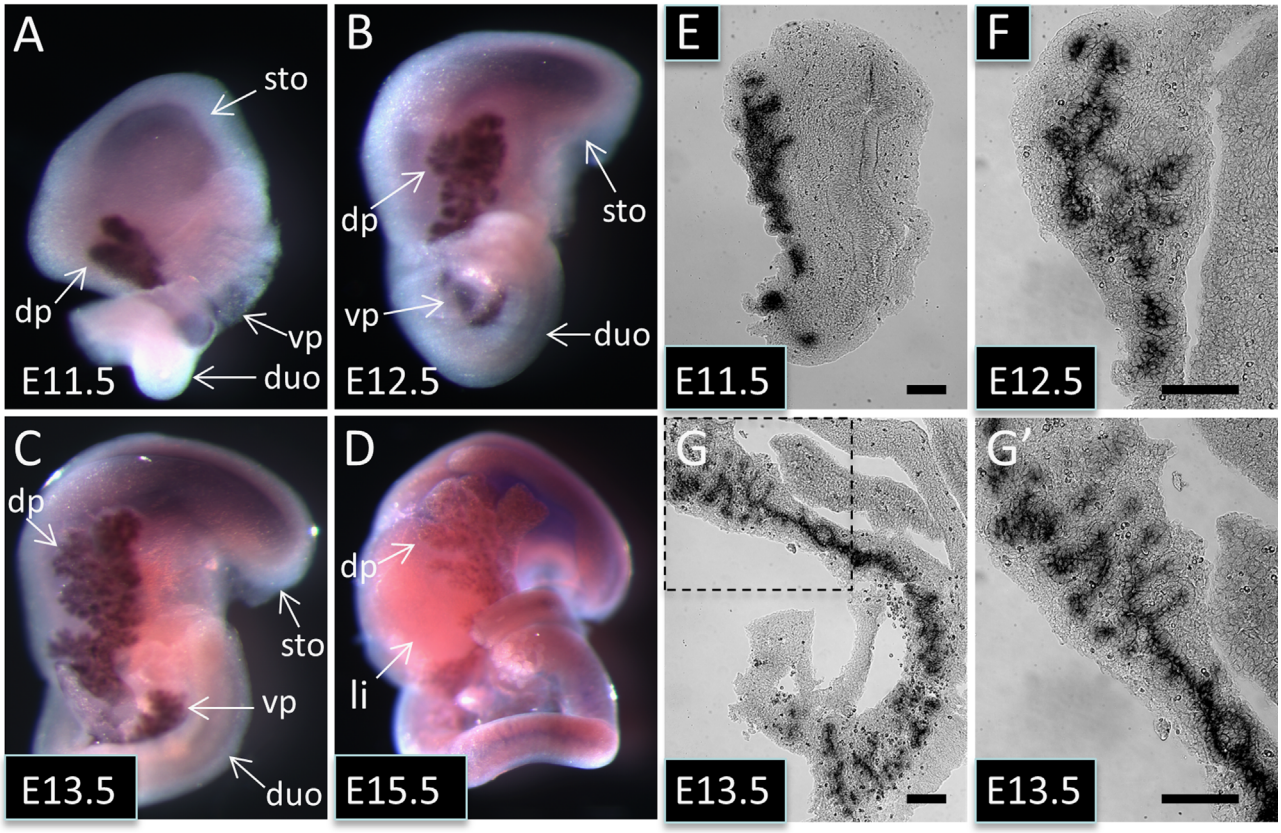

Fig. 1. Expression profile of *Wnt7b* during pancreas development. (A–D)Whole mount *in situ* hybridization analysis of the expression pattern of *Wnt7b* at the indicated developmental stages. (E–H) Sections of whole mount *in situ* hybridization at E11.5 (E), E12.5 (F) and E13.5 (G, G') depicting epithelial restricted expression of *Wnt7b*. High magnification of the boxed area in (G) is shown in (G'). Sto: stomach; dp: dorsal pancreas; vp: ventral pancreas; duo: duodenum; li: liver. Scale bar: 100 μm.

assess pancreatic progenitor cell mass in WT and *Wnt7b* PKO embryos over a range of developmental stages beginning at organ specification. By morphometry, we detected no difference in Pdx1$^+$ pancreatic progenitor cell numbers between WT and *Wnt7b* PKO embryos at E9.5 and E10.5, and the overall bud size was comparable. We concluded that pancreatic fate specification occurred normally in *Wnt7b* PKO embryos (data not shown). At E11.5, we noted a reduction in pancreatic progenitor mass of *Wnt7b* PKO embryos, though not statistically significant (Fig. 4A–C). We then focused our analysis at E13.5 which marks the onset of the secondary transition stage of pancreas development. We noted a more pronounced reduction in pancreatic progenitor cell mass at E13.5 compared to that observed at E11.5 (Fig. 4E–G). A possible explanation for the hypoplasia in *Wnt7b* null pancreas could be apoptosis. However, TUNEL assay performed at various developmental stages showed no difference in apoptosis between WT and *Wnt7b* PKO pancreas, this being essentially non-existent in both conditions (data not shown). We analyzed cell proliferation rate at E11.5 and E13.5 using antibodies against the M-phase marker phospho-histone H3 (pHH3). A significant reduction in the proliferation rate of pancreatic progenitor cells was observed at both E11.5 (Fig. 4A, B, and D) and E13.5 (Fig. 4E, F, and H). Based on the above observations we conclude that *Wnt7b* is required for proper organ size determination by controlling pancreatic progenitor cell proliferation prior to, and up to the secondary transition.

Elevated expression of Wnt7b results in formation of polycystic duct-like structures

We and others have previously shown that *Fgf10* plays an important role in pancreatic progenitor cell expansion (Bhushan et al., 2001), and Fgf10 is able to maintain cells in a progenitor state when overexpressed (Hart et al., 2003; Norgaard et al., 2003). Given the requirement of Wnt7b in pancreatic progenitor cell proliferation, we set out to test if elevation of Wnt7b would maintain the progenitor cell state and/or cause hyperproliferation. The full-length coding sequence of human *Wnt7b* (which has 99%, 345/349, protein sequence identity to mouse Wnt7b) was inserted downstream of a tetracycline inducible promoter (pTRE) followed by an IRES-nEGFP sequence to aid tracking of Wnt7b-expressing cells. The construct was injected into fertilized oocytes to generate transgenic founders which were subsequently mated to Pdx1-tTA knock in (Pdx1-tTA^{KI}) mice (Holland et al., 2005) to allow expression of the transgene in the developing pancreas, duodenum and antral stomach, in the absence of doxycycline. Expression of the transgene was easily visualized by EGFP expression (Fig. 5A and B). We identified three founder strains displaying EGFP expression in the developing pancreas and duodenum, and all generated comparable phenotypes. pTRE2-*Wnt7b*-IRES-nEGFP/Pdx1-tTA^{KI} double transgenic mice ("DTG") developed an enlarged antral stomach and a dorsal pancreatic bud that appears enlarged and cystic (Fig. 5A and B). Single-transgenic embryos for either pTRE2-*Wnt7b*-IRES-nEGFP or Pdx1-tTA^{KI} displayed no EGFP expression and had no apparent pancreatic abnormalities at all embryonic stages examined. In the WT pancreas expression of Hnf1β, Sox9 and Hnf6 become restricted to ductal cells by E16.5 (Fig. 5C, and E). Histological analysis at E16.5 and E18.5 revealed that *Wnt7b* DTG pancreata consisted predominantly of polycystic duct-like complexes that stained positive for Hnf1β, Sox9, Hnf6 and the duct-specific lectin DBA (Fig. 5C–J, data not shown). Compared to the distended WT pancreatic ducts, the duct-like structures of the *Wnt7b* DTG pancreas were extremely dilated (Fig. 5D, F, H, and J). In contrast to the abundant duct-like structures, a very few amylase$^+$ acinar cells were detected in the *Wnt7b* DTG pancreas (Fig. 5J). When observed,

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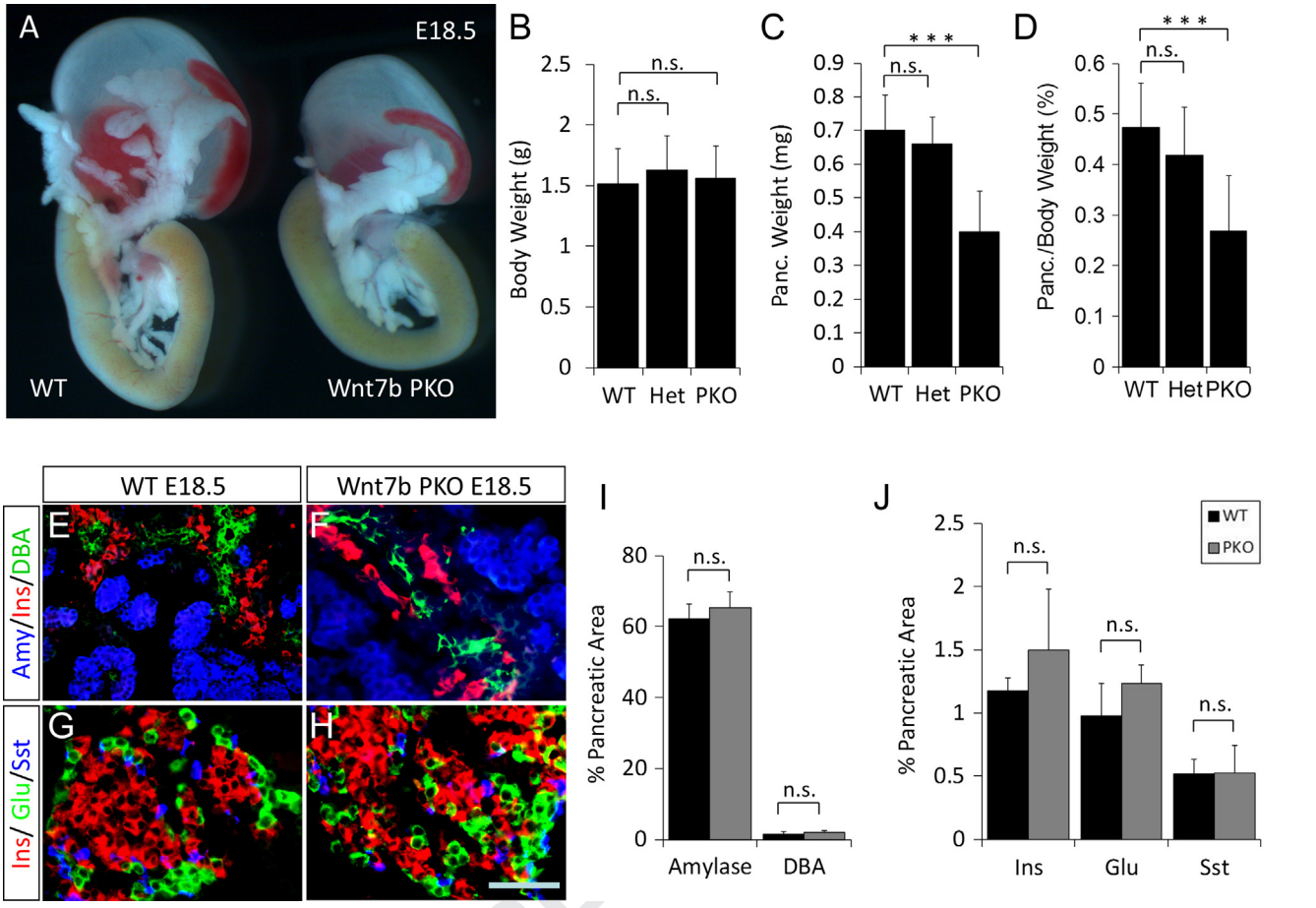

Fig. 2. *Wnt7b* mutant mice develop a hypoplastic pancreas. (A) Morphological view of mdigut from wild type (WT) and pancreas specific *Wnt7b* knock out (*Wnt7b* PKO) embryos at E18.5. (B–D) Quantification by weight of: (B) embryonic pancreatic tissue; (C) ratio of pancreatic weight to whole body weight of WT, heterozygous and; (D) *Wnt7b* knock out embryos at E18.5. (E–F) Immunofluorescence staining of amylase (Amy), insulin (Ins) and duct specific lectin (DBA) in WT (E) and *Wnt7b* knock out embryos (F) at E18.5. (G–H) Immunofluorescence staining of insulin, glucagon and somatostatin in WT (G) and knock out (H) embryos at E18.5. (I–J) Morphometric quantification of amylase and DBA (I) and insulin, glucagon and somatostatin (J). Graphs values are mean ± s.d.; n.s: not significant; ***$p < 0.001$. Scale bar: 50 μm.

such were located at the periphery of the duct-like metaplastic structures (Fig. 5J). Endocrine cell fate development was likewise severely attenuated in the *Wnt7b* DTG pancreas (Fig. 5I, and J), which included all endocrine sub-types (data not shown).

To gain further insight into the effect of Wnt7b-overexpression on the embryonic pancreas, we performed genomics-based expression profiling (Illumina mRef.) comparing Wnt7b overexpressing pancreas to WT littermates ($n=3$/condition, at E14.5). A false discovery rate-limited (FDR < 0.1) gene list was compiled, extracting genes that were >2 fold increased/decreased in the Wnt7b-overexpressing pancreas. Principal component analysis validated the conditions and samples to be individually grouped. This analysis validated the global reduction in expression of genes associated with terminal endocrine fates, revealing a typical reduction to 10–30% of WT expression of genes including *Ins1*, *Ins2*, *Gcg*, *Sst*, *Abcc8*, and *Chga* (Supplementary Fig. 4A). Similarly, a general reduction to 20–40% of WT expression of acinar-specific genes (*e.g. Amy2*, *Ctrb1*, *Ela1*, *Ela2*, *Ptf1a*, *Rbpjl*, *bHLHb8* (*Mist1*)) and others was also observed (Supplementary Fig. 4B).

Wnt7b overexpression suppresses both endocrine and acinar progenitor fate assignment

To characterize the observed effects of pancreatic *Wnt7b* overexpression in more detail, we addressed the expression of markers of various pancreatic progenitor cell populations. At E13.5 the pancreatic progenitor marker Pdx1 is expressed broadly in all pancreatic epithelial cells, outlining the structural organization of the pancreatic epithelium (Fig. 6A, D, G, and J; Zhou et al., 2007). In the *Wnt7b* DTG pancreas, Pdx1 was expressed comparatively to WT; however, the pancreatic epithelium failed to undergo branching morphogenesis with lack of well-defined tips (Fig. 6B, E, H, and K). Accordingly, Ptf1a, which is normally restricted to the distal tips (TipPC) in WT (Fig. 6D) (Cockell et al., 1989; Hald et al., 2008; Krapp et al., 1996) was reduced in the *Wnt7b* DTG pancreas (Fig. 6E, and F). The loss of TipPCs was accompanied by a loss of TrPCs, as the TrPC transcription factor Nkx6.1 was almost abolished in *Wnt7b* DTGs (Fig. 6A–C). Within the TrPC field, all pancreatic endocrine cells are derived from Neurogenin3 (Ngn3) positive progenitor cells (Gradwohl et al., 2000; Gu et al., 2002). At both E12.5 and E13.5 Ngn3 expression was almost abrogated in the *Wnt7b* DTG pancreas (Fig. 6G–I and data not shown). We conclude that lack of formation of endocrine and acinar precursor cells is the reason for the blunted differentiation of the more terminal state (Fig. 5G, and H).

The above findings suggested that Wnt7b overexpression suppressed the formation of both endocrine and acinar lineages with progenitor cells assuming a duct-like phenotype, but when this occurred remained unclear. We therefore examined earlier developmental stages. At E12.5 both WT and DTG pancreata display minimal but comparable levels of DBA staining arguing against premature ductal cell formation (data not shown). However, corresponding to the formation of terminally differentiated ductal cells at E13.5, luminal DBA reactivity was detectable within the WT pancreatic epithelium (Fig. 6J). In contrast, DBA$^+$ epithelial cells were far more abundant in *Wnt7b* DTG epithelium (Fig. 6K and L) and DBA reactivity

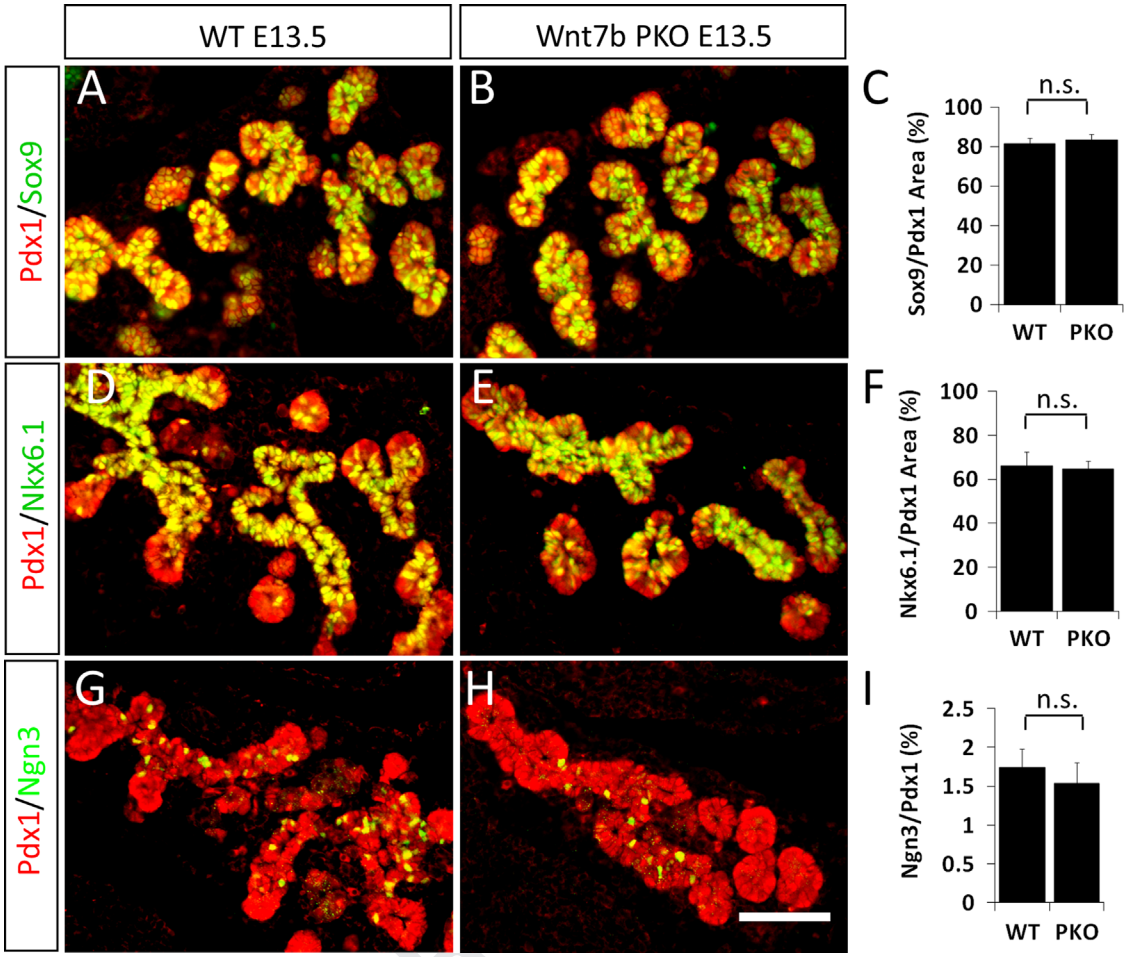

Fig. 3. Patterning of the embryonic pancreas is unaffected by *Wnt7b* mutation. (A, B) Immunofluorescence staining of Pdx1 and Sox9 in WT (A) and *Wnt7b* knock out pancreas (B). (C) Morphometric quantification of the percentage of Pdx1 epithelial cells that express Sox9. (D, E) Immunofluorescence staining of Pdx1 and Nkx6.1 in WT (D) and *Wnt7b* PKO pancreas (E). Morphometric analysis of the percentage of Pdx1 cells that express Nk6.1 in the WT and *Wnt7* PKO pancreas (F). (G, H) Immunofluorescence staining of Pdx1 and Ngn3 in WT (G) and *Wnt7b* PKO pancreas (H). Morphometric analysis of the percentage of Pdx1 cells that express Nkx6.1 in the WT and *Wnt7b* PKO pancreas (I). Scale bar: 50 μm.

was present on the apical and baso-lateral surfaces of the *Wnt7b* DTG pancreatic epithelium (Fig. 6K). These observations suggest that at time of ductal cell formation (E12.5–E13.5) elevation of Wnt7b directs pancreatic progenitor cells almost exclusively into the ductal lineage at the expense of other lineages.

Lack of primary cilia has been shown to cause cyst formation in the pancreas (Cano et al., 2006). Given that the *Wnt7b* DTG pancreas develops polycystic duct-like structures, we examined the state of primary cilia through immunostaining for acetylated tubulin. Compared to the WT pancreas where primary cilia line the luminal side of the epithelial cords (Supplementary Fig. 5A, A', C, and C'), we notice a dramatic loss of primary cilia within the cystic duct-like structures of the *Wnt7b* DTG pancreas (Supplementary Fig. 5B, B', D, and D'). Loss of primary cilia has been associated with deregulated signaling leading to defective pancreas development. Of note, in the absence of primary cilia the pancreatic epithelium is susceptible to hedgehog signal hyperactivation leading to polycystic ductal structures similar to that observed in the Wnt7b-overexpressing pancreas (Cervantes et al., 2010). Consistent with this, microarray analysis indicated an increased expression of the hedgehog target gene *Gli2* in the *Wnt7b* DTG pancreas (Supplementary Table 1).

Overexpression of Wnt7b at the onset of pancreas development disrupts pancreatic morphogenesis and differentiation

We reasoned that the effect of *Wnt7b* overexpression on the pancreas may either be due to the continuous persistent expression of the transgene, or that this may arise from a specific developmental time point during which the pancreatic epithelium is sensitive to Wnt7b protein levels. To clarify this we limited expression of the transgene to a brief time window, between the onset of Pdx1 promoter activation (~E8.0) till E11.5 (*Wnt7b DTG Brief ON*). In the presence of doxycycline, the transactivating protein tTA fails to bind and activates transcription from the pTRE promoter leading to loss of transgene expression. We switched off expression of the transgene by administering doxycline to pregnant females starting from E11.5 until E16.5, at which point embryonic pancreatic tissue was analyzed (Fig. 7A). Transgenic expression of EGFP was lost in *Wnt7b* DTG embryos, indicating inactivation of transgene expression. Remarkably, transient Wnt7b overexpressing pancreas was similar to that observed when Wnt7b was expressed continuously (Fig. 7B, and C; Fig. 5I, and J). *Wnt7b DTG Brief ON* pancreata displayed epithelial dysmorphogenesis, maintained expression of the ductal marker DBA, and failed to express terminal differentiation markers including insulin (Fig. 7B, and C). We next limited the activation of the transgene from the onset of pancreas development until E10.5, and also until E9.5 (Fig. 7A). Surprisingly, these very brief exposures to exogenous Wnt7b were sufficient to induce pancreatic epithelial dysmorphogenesis similar to continuous transgene activation starting from the onset of pancreas development (Fig. 7D, and E). However, by limiting expression to the window of E8.0-E9.5, endocrine cell differentiation as marked by the expression of insulin and glucagon was rescued (Fig. 7F, and G, and data not shown), yet, epithelial dysplasia was still observed. These

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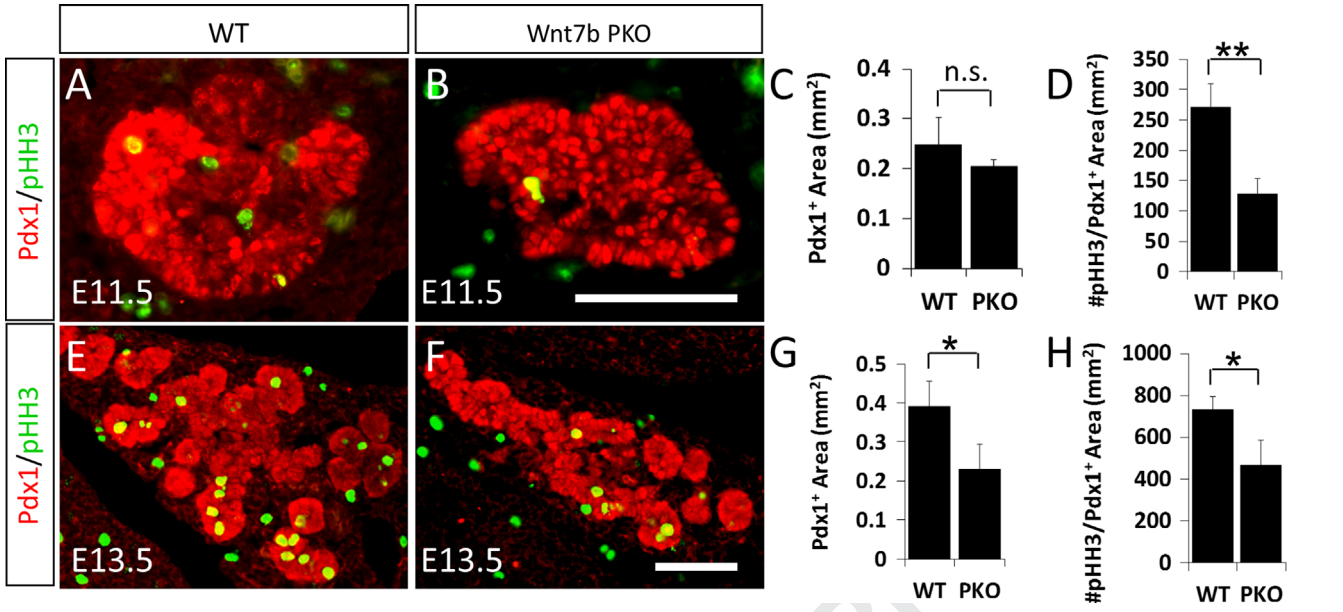

Fig. 4. *Wnt7b* deletion leads to decreased proliferation rate of pancreatic progenitor cells. (A–D) *Wnt7b* deletion results in reduced proliferation rate of pancreatic cells at E11.5 but not a significant reduction in epithelial mass. (A,B) Immunofluorescence staining of Pdx1 and phospho-histone H3 in WT (A) and *Wnt7b* PKO pancreas (B) at E11.5. (C) Morphometric quantification of pancreatic mass between WT and *Wnt7b* PKO embryonic pancreas at E11.5. (D) Morphometrical quantification of the proliferation rate of WT and *Wnt7b* PKO pancreas at E11.5. (E, F) Immunofluorescence staining of Pdx1, desmin and phospho-histone H3 in E13.5 WT (E) and *Wnt7b* PKO (F). (G) Morphometric quantification of pancreatic mass in WT and *Wnt7b* PKO. (H) Morphometric quantification of the proliferative rate of WT and *Wnt7b* PKO pancreas at E13.5. Graphs values are mean ± s.d.; n.s.: not statistically significant; $^{*}p < 0.05$; $^{**}p < 0.01$. Scale bar: 50 μm.

observations suggest that the epithelial dysmorphogenesis resulting from Wnt7b overexpression emanates from the early pancreatic progenitor stage, prior to the onset of branching morphogenesis.

Overexpression of Wnt7b during the secondary transition stage of pancreas development induces a disproportionate increase in mesenchyme, but decrease in acinar mass

The outcome of the *Wnt7b DTG Brief ON* experiment suggests that the early pancreas is highly sensitive to Wnt7b expression levels. Also, as endogenous Wnt7b promotes pancreatic progenitor cell proliferation starting from E11.5 (Fig. 4A–D), we reasoned that delayed activation of the *Wnt7b* transgene, starting from E11.5 (Fig. 8A), would: (i) circumvent the deleterious effect of exogenous Wnt7b in the early pancreatic epithelium, and (ii) lead to increased organ size. Contrary to our prediction, delayed Wnt7b overexpression (*Wnt7b DTG Delayed ON*) resulted in a smaller pancreatic mass than that of WT (Fig. 8B–D). Though smaller, immunofluorescence analysis revealed that the *Wnt7b DTG Delayed ON* pancreas contains terminally differentiated cells of endocrine, acinar and ductal lineages (Fig. 8E, and F). Morphometric analysis revealed a decrease in the percentage of acinar cells relative to total pancreatic mass, while insulin and duct cells remain comparable to that of WT (Fig. 8I). Contrary to acinar cell mass, we observed a two-fold increase in pancreatic mesenchyme relative to total pancreatic mass, in the *Wnt7b DTG Delayed ON* (Fig. 8G–I). This suggests that the effect of Wnt7b is not limited to the epithelium, but involves effects in the pancreatic mesenchyme.

Epithelially-expressed Wnt7b induces mesodermal gene expression and the expansion of a specific pancreatic mesodermal subset of cells

The increased mesenchymal mass in the *Wnt7b DTG Delayed ON* pancreas prompted us to investigate the effect of continuous Wnt7b overexpression on the mesenchyme. Because pancreatic mesenchyme is the source of various growth and differentiation inducing factors, the impact of Wnt7b originating from the epithelium and sensed by the mesenchyme could plausibly be modifying the mesenchymal signaling and reciprocally impact the epithelial compartment. Immunofluorescence analysis with antibodies to various mesenchymal marker genes revealed an increased mesenchymal mass in the *Wnt7b* DTG pancreas (Supplementary Fig. 6A–D). In addition to increased expression of desmin (Supplementary Fig. 6A, and B), we detected a dramatic increase in the expression of cFos in the *Wnt7b* DTG mesenchyme (Supplementary Fig. 6C, and D). cFos is a MAPK target and a component of the AP-1 complex commonly linked to proliferation. Thus, cFos may play a role in the increased mass of the mesenchyme. Interestingly, we did not detect any significant difference in the pancreatic mesenchymal mass or proliferation rate in Wnt7bKO embryos (Supplementary Fig. 6 E–G). This suggests that the increased mesenchymal mass in the Wnt7b DTG is the result of hyperactivation of Wnt signaling.

Analysis of the genomic profile of the *Wnt7b* DTG pancreas at E14.5 revealed increased expression of several mesodermal-cell specific genes which encode extracellular matrix proteins (*e.g. Col6A1*, *Col16A1*, *Col1A2*, *Dcn*, *Lox*, *Lum*, *Fhl1*, and *Fbln1*) (Supp. Table 1). These genes represented a generalized 2–3 fold increased expression compared to WT. The increased expression of a larger mesodermal subset of genes correlates well with the relative increase in abundance of pancreatic mesenchymal mass. Because the genomics analysis does not provide information of compartment-specific gene expression, we sought to stratify the Wnt7b-induced genes by comparing to available data sets in which epithelial and mesenchymal separation was performed. Expression data based on microdissected E10.5 pancreatic mesenchyme and epithelium was available on the Affymetrix MGU133 platform and we compared such to the upregulated list. We found approx. 80% of the Wnt7b upregulated genes to be highly enriched in pancreatic mesenchyme, and a much smaller subset enriched in pancreatic epithelium (Supplementary Table 2). Similarly, when using an available dataset based on isolated intestinal epithelium and mesenchyme, we also noted a strong enrichment of Wnt7b-upregulated genes in the mesenchymal, rather than epithelial tissue of the intestine (Supplementary Table 2). Ingenuity™-based pathway analysis provided more information on the Wnt7b

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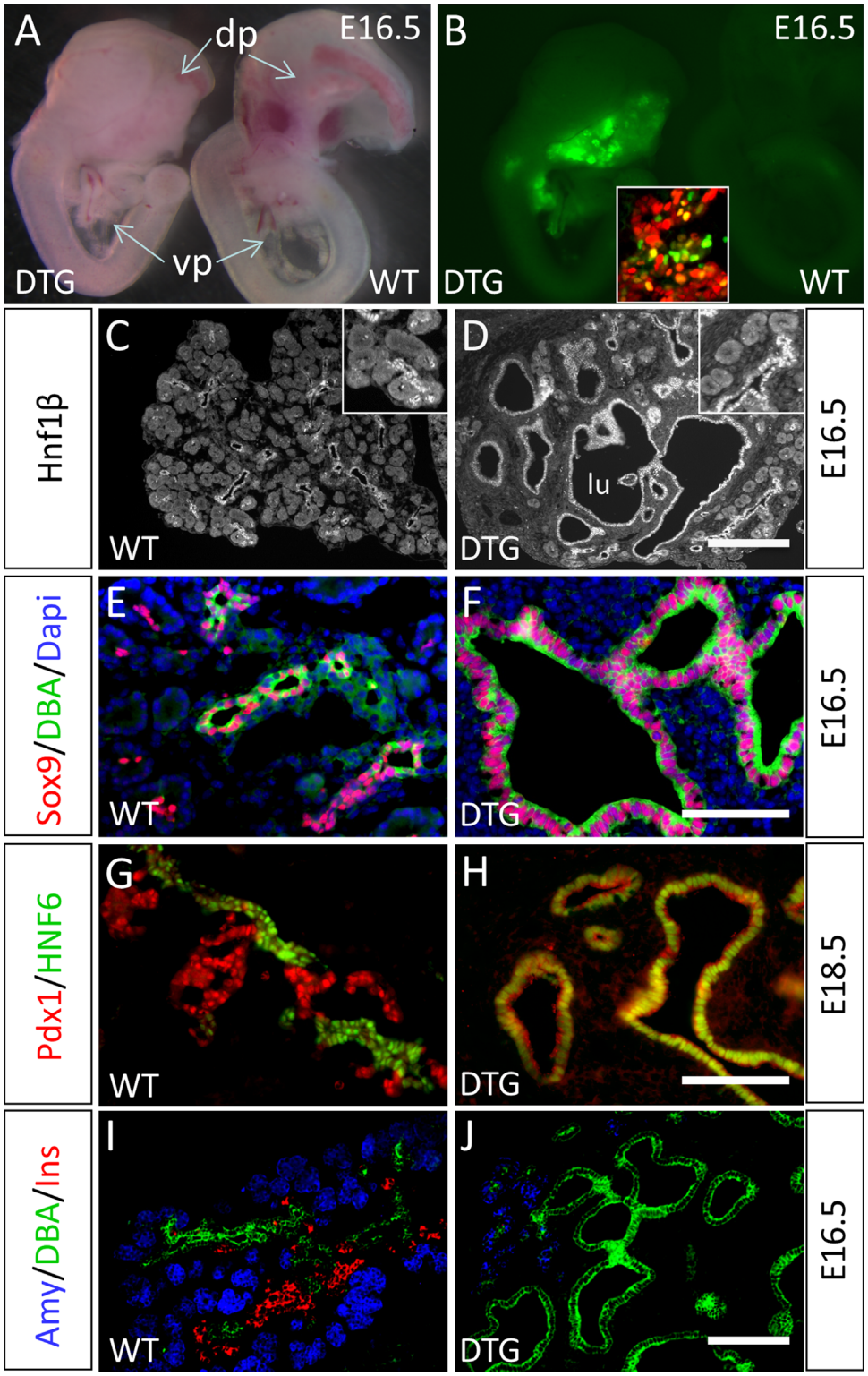

Fig. 5. Overexpression of Wnt7b leads to the development of polycystic duct-like structures. (A) Mid-gut including stomach, duodenum and pancreas of pTRE2-*Wnt7b*-IRES-nEGFP; Pdx1-tTA^{KI} double transgenic (DTG) pancreas and WT pancreas in bright field. (B) Fluorescence view of DTG and WT mid-gut. The presence of nEGFP depicts the expression of the transgene in *Wnt7b* DTG pancreas. Inset in panel B shows mosaic expression of the transgene (EGFP) within the pancreatic epithelium (stained red for Pdx1). (C, D) Immunofluorescence staining of Hnf1β in WT (C) and *Wnt7b* DTG pancreas (D); Sox9, DBA and Dapi in WT (E) and DTG (F); Pdx1 and Hnf6 in WT (G) and *Wnt7b* DTG (H); amylase, DBA and insulin in WT (I) and *Wnt7b* DTG (J). Scale bar: 50 μm.

upregulated transcript pool, helping to identify larger network of multiple ECM associated genes, proteases and inhibitors involved in ECM remodeling, lysyl oxidases and netrins (Supplementary Fig. 7). Several Wnt-pathway inhibitors were increased, including *Dkk3*, *Sostdc1*, and *Wif1* (Supplementary Table 1, Supplementary Fig. 7), possibly representing feedback inhibition. Interestingly, several genes encoding growth factors were increased in response to elevated Wnt7b, including *Fgf7*, *InhbA* (encoding Activin A), and *Bmp4* (Supplementary Table 1). These results suggest that Wnt7b elicits dramatic changes to

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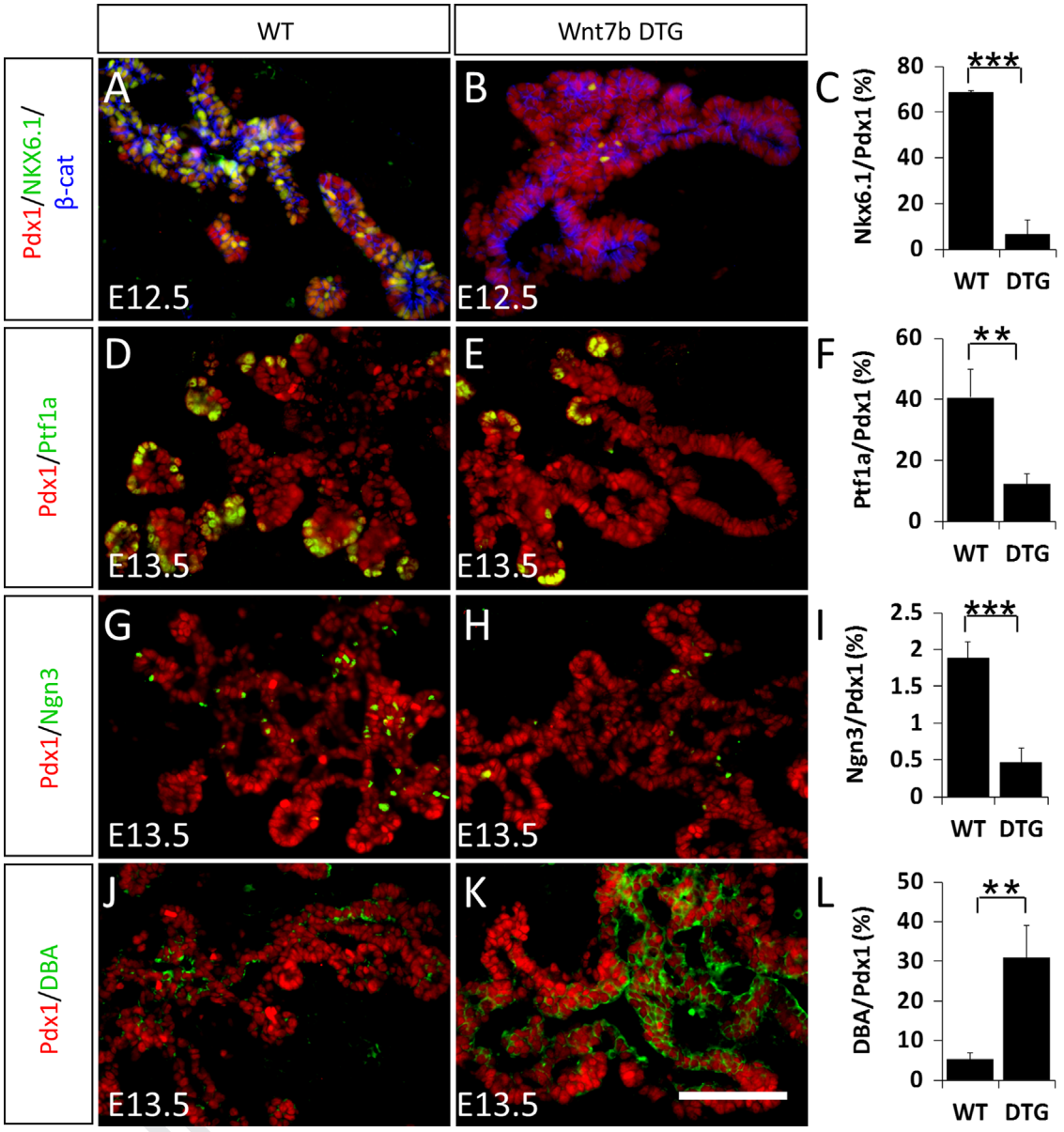

Fig. 6. Wnt7b overexpression leads to suppression in both tip and trunk progenitor gene expression. (A, B) Immunofluorescence staining of Pdx1, Nkx6.1 and β-catenin in WT (A) and *Wnt7b* DTG (B) pancreas. (C) Morphometric quantification of the percentage of Pdx1$^+$ cells that express Nkx6.1. (D, E) Immunofluorescence staining of Pdx1 and Ptf1a in WT (D) and *Wnt7b* DTG pancreas (E). (F) Morphometric quantification of the percentage of Pdx1$^+$ cell that express Ptf1a. (G, H) Immunofluorescence staining of Pdx1 and Ngn3 in WT (G) and *Wnt7b* DTG pancreas (H). (I) Morphometric quantification of the percentage of Pdx1$^+$ cells that express Ngn3. Immunofluorescence staining of the expression of Pdx1 and DBA in WT and *Wnt7b* DTG pancreas. (L) Morphometric quantification of the area of DBA normalized to Pdx1$^+$ cells. Graphs values are mean ± s. d. $^{**}p < 0.01$; $^{***}p < 0.001$. Scale bar: 50 μm.

the pancreatic signaling environment, and creates a compound phenotype that manifests in both germ layer components.

Analysis of the microarray data of the *Wnt7b* DTG pancreas indicated upregulated expression of several canonical Wnt target genes such as Axin2, Pitx2, Apcdd1, and Ednra [Supplementary Table 1, (Zirn et al., 2006)]. This suggests that Wnt7b operates through canonical Wnt signaling and prompted us to conduct histological analysis to define which cells are directly responsive to Wnt7b. The canonical Wnt target gene Lef1 is present predominantly in the pancreatic epithelium at E10.5 and becomes expressed in both the epithelium and mesenchyme by E13.5 (Supplementary Fig. 8). We detect a strong upregulated expression of Lef1 specifically within the pancreatic mesenchyme in the Wnt7b DTG pancreas. Interestingly, this is also accompanied by reduced Lef1 expression within the epithelial compartment (Fig. 9A–F). Conversely in the Wnt7b PKO pancreas, Lef1 expression is lost in the mesenchyme and reduced within the pancreatic epithelium (Fig. 9G–L). Taken together, this evidence suggests that Wnt7b operates via canonical Wnt signaling to both the pancreatic epithelium and mesenchyme during pancreas development.

Discussion

A number of studies have implicated the canonical Wnt signaling pathway as critical for pancreatic progenitor cell growth. But, as these studies have been based predominantly on genetic perturbations of down-stream Wnt effector genes such as β-catenin, the identity of the Wnt ligand(s) responsible for Wnt pathway activation and the tissue of origin have not been determined until now. In

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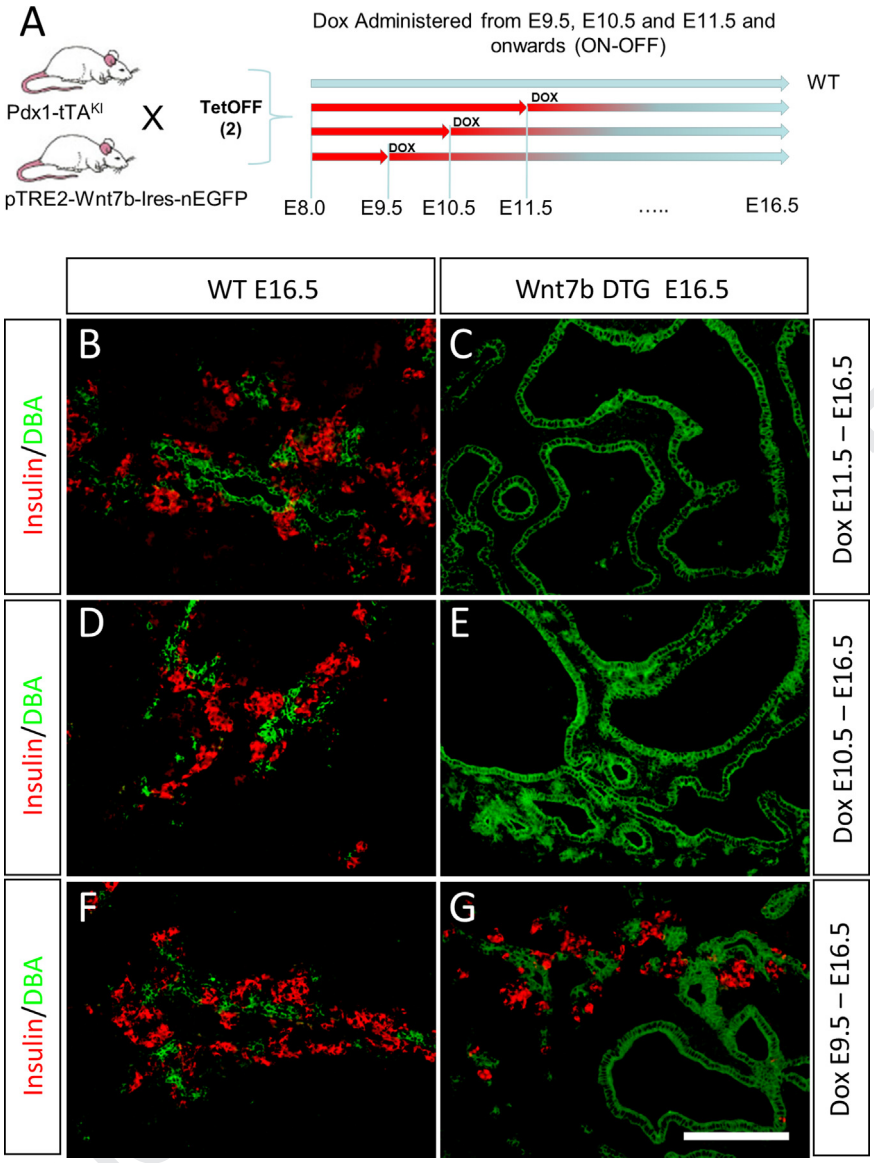

Fig. 7. Early overexpression of *Wnt7b* disrupts epithelial morphogenesis and differentiation. (A) Schematic of time points of doxycycline administration to shut off transgene expression. (B, C) Insulin and DBA immunostaining in WT and *Wnt7b* DTG at E16.5 following doxycycline administration to pregnant females between E11.5 and E16.5. (D, E) Expression of insulin and DBA in WT (D) and *Wnt7b* DTG pancreas (E) at E16.5 from pregnant females given doxyclycline from E10.5 to E16.5. (F, G) Insulin and DBA expression in E16.5 WT (F) and *Wnt7b* DTG pancreas derived from pregnant females which were provided doxycycline at E9.5 till E16.5. Scale bar: 50 μm.

this study we have identified Wnt7b as a critical canonical Wnt ligand expressed in the epithelium and required for pancreatic progenitor cell growth. We find that pancreas-specific deletion of Wnt7b leads to reduced proliferation of pancreatic progenitor cells just prior to and during the secondary transition stage of pancreas development, with a concomitant pancreatic hypoplasia. Interestingly, however, overexpression of Wnt7b under the Pdx1 promoter does not lead to increased pancreatic progenitor mass, as would be expected from the loss-of-function studies; instead this results in suppression of endocrine and acinar fate differentiation accompanied by polycystic duct-like epithelial complexes and increased pancreatic mesenchymal mass.

Wnt signaling in pancreatic progenitor cell growth

Multiple Wnt ligands have been reported in the pancreas (Heller et al., 2003). Yet their individual roles in pancreas development have not been defined. Broad spectrum suppression of Wnt signaling at the receptor level through overexpression of a diffusible dominant-negative frizzled 8 receptor fragment (Frz8CRD) leads to reduced pancreatic mass (Papadopoulou and Edlund, 2005). Also targeted deletion of Wntless in the pancreatic epithelium leading to defective secretion of Wnt ligands from the pancreatic epithelium results in reduced pancreatic progenitor growth (Carpenter et al., 2010). These reports, together with the observation that targeted deletion of Wnt7b leads to a significant reduction in pancreatic progenitor mass, suggest that Wnt7b is a major canonical Wnt ligand for pancreatic progenitor cell growth. Studies involving targeted deletion of downstream canonical Wnt signaling effectors such as β-catenin or pygopus2 (Baumgartner et al., 2014; Jonckheere et al., 2008; Murtaugh et al., 2005; Wells et al., 2007) have also yielded reduced pancreatic mass, supporting a role for canonical Wnt signaling in pancreatic progenitor growth. However, epithelial specific β-catenin deletion also leads to defects in acinar fate differentiation (Baumgartner et al., 2014; Murtaugh et al., 2005; Wells et al., 2007). We do not observe defects in acinar cell differentiation following pancreas specific deletion of Wnt7b. This raises the possibility that the acinar defects in the β-catenin deficient pancreas

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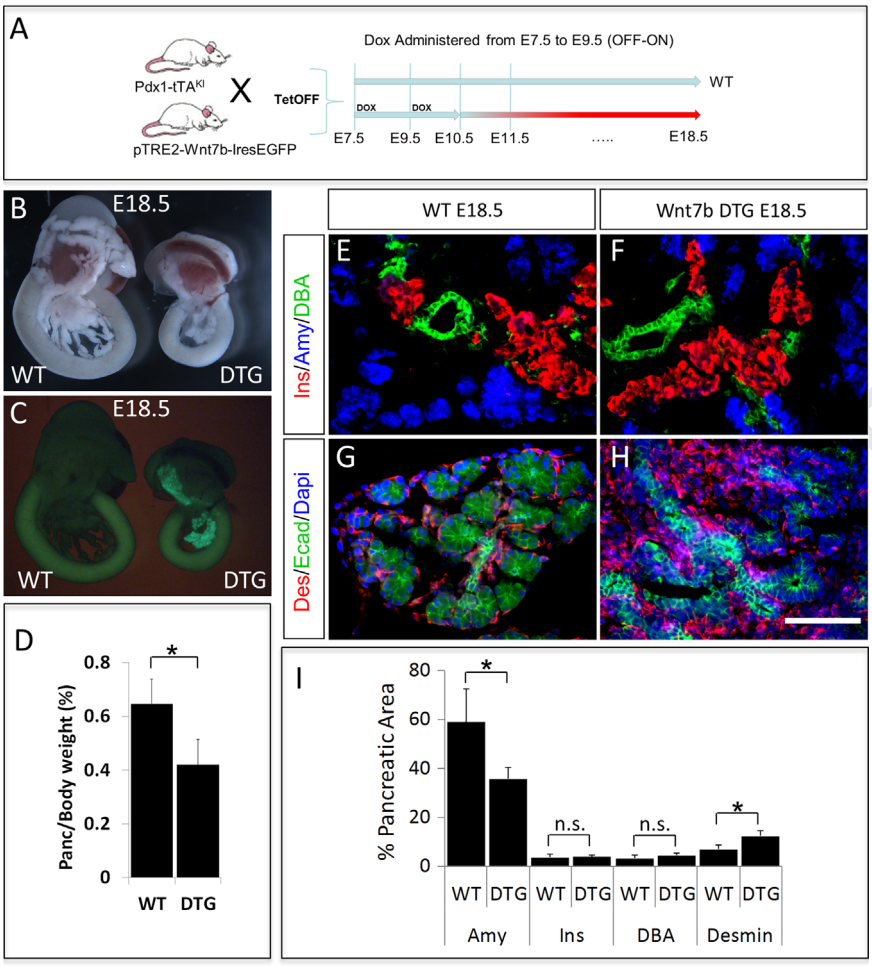

Fig. 8. Overexpression of *Wnt7b* during mid-pancreas development leads to decreased total pancreatic mass but a disproportionate increase in the mesenchyme. (A) Schematic of doxycycline administration to suppress *Wnt7b* expression at the onset of pancreas development (*Wnt7b Delayed ON*). (B) Bright field view of WT and *Wnt7b Delayed ON* mid-gut including pancreas at E18.5. (C) Fluorescent image of panel B depicting expression of the transgene via nEGFP in the *Wnt7b Delayed ON DTG* pancreas. (D) Ratio of pancreas to body weight in WT and *Wnt7b Delayed ON* DTG at E18.5. Immunofluorescence staining of WT and *Wnt7b Delayed ON DTG* for insulin, amylase and DBA (E, F); desmin, E-cadherin, and nuclear dapi (G, H). (I) Morphometrical quantification of the percentage of amylase, insulin, DBA and desmin positive cells relative to total pancreatic area in WT and *Wnt7b Delayed ON DTG*. Graphs values are mean ± s.d.; n.s: not significant; $^*p < 0.05$. Scale bar: 50 μm.

might be due to functions of β-catenin that are independent of Wnt signaling, such as a role of β-catenin as part of cell adhesion complex (Dessimoz et al., 2005).

Window of competence for canonical Wnt signaling in progenitor growth

Based on the outcome of the *Wnt7b* deletion studies, we tested the extent to which pancreatic mass can be increased through overexpression of exogenous Wnt7b in the pancreatic epithelium, under the control of the Pdx1-promoter. Rather than inducing hyperproliferation, the overexpression of Wnt7b in the pancreas led to pancreatic epithelial dysmorphogenesis, in which both endocrine and acinar fates were suppressed. By temporally restricting the exogenous Wnt7b overexpression to only brief developmental time widows, we established that the observed pancreatic epithelial dysplasia results from the early overexpression of Wnt7b (~E8.5 to E11.5), and not due to the continuous Wnt7b hyperactivation, which mainly affects mesenchymal cells in the organ. This suggests that the early pancreatic endoderm is sensitive to canonical Wnt signaling levels, while the pancreatic mesenchyme remains Wnt-responsive later. Pancreatic epithelial-restricted overexpression of β-catenin yields similar defects in the pancreatic epithelium, when the onset of β-catenin expression is at the early stages of pancreas development (Heiser et al., 2006). Although Wnt signaling is required for the early induction of definitive endoderm (Mfopou et al., 2014), canonical Wnt signaling activity patterns the early endoderm towards posterior fates, at the expense of foregut endodermal fates such as the pancreas (McLin et al., 2007; Rodriguez-Seguel et al., 2013). Our analysis of the early pancreas suggests that early onset of Wnt7b expression inhibits the patterning of multi-potent pancreatic progenitor cells into "tip" and "trunk" domains and thus abrogates subsequent differentiation into acinar and endocrine lineages.

Surprisingly, delaying the onset of Wnt7b overexpression to E11.5 leads to pancreatic hypoplasia characterized by a decrease in acinar cell mass while the mesenchymal compartment increases relative to total pancreatic mass. This is consistent with our observation that the pancreatic mesenchyme is a direct target of Wnt7b from the pancreatic epithelium. Though targeted deletion of Wnt7b causes a reduction in pancreatic progenitor growth, we did not detect an increased progenitor growth following overexpression of Wnt7b *in vivo*. Given that the mesenchyme is a direct target of Wnt7b, it is likely that Wnt7b overexpression transforms the mesenchyme, which in turn has negative effect on the epithelium, but this remains to be established. The decreased expression of Lef1 within the pancreatic epithelium of Wnt7b DTG may contribute to the reduced epithelial mass. Our future studies are aimed at culturing Wnt7b overexpressing epithelial cells in the absence of

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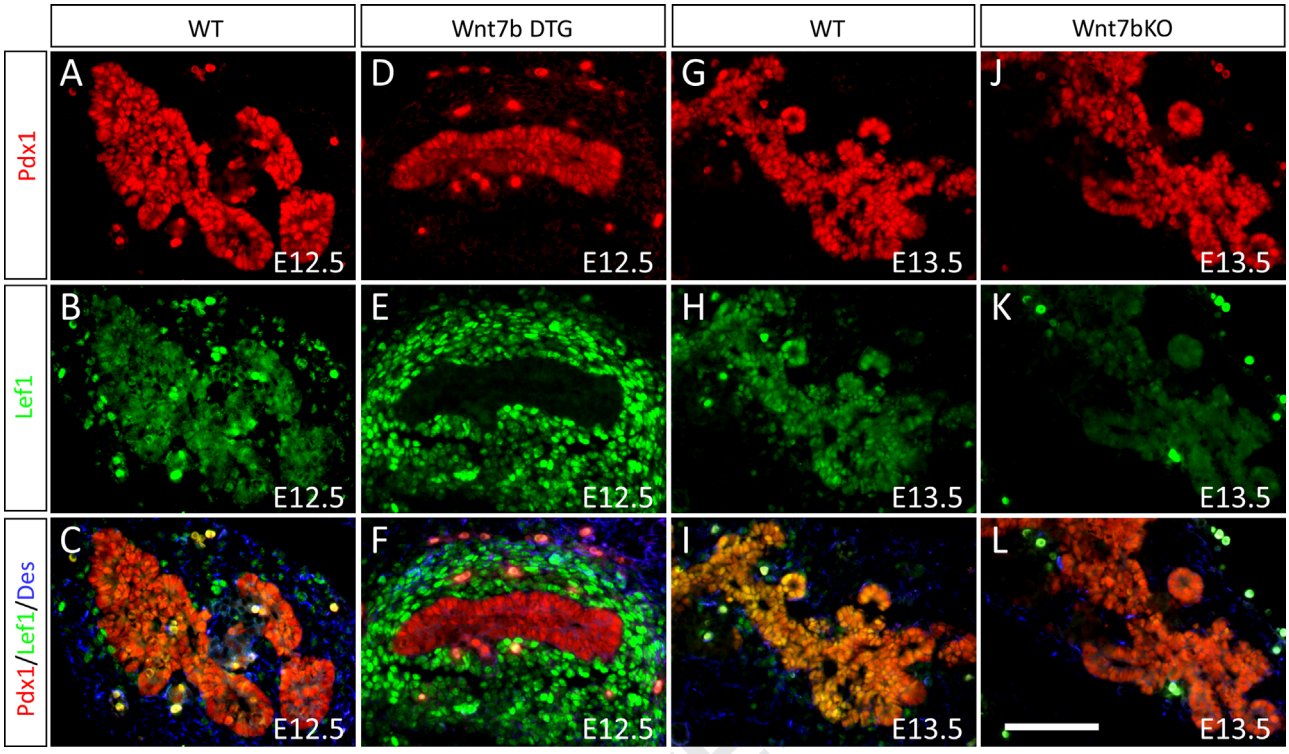

Fig. 9. Pancreatic epithelial specific Wnt7b controls the expression of the canonical Wnt target Lef1 in both pancreatic epithelium and mesenchyme. (A–F) Wnt7b overexpression leads to hyperactivation of Lef1 expression specifically within the pancreatic mesenchyme. Immunofluorescence staining of Pdx1 (A, D), Lef1 (B, E) and overlay of Pdx1, Lef1 and Desmin (C, F) in E12.5 WT (A–C) and Wnt7b DTG pancreas (D–F). (G–L) Pancreatic epithelial deletion of Wnt7b leads to loss of Lef1 within the pancreatic mesenchyme and reduced expression within the epithelial compartment. Immunofluorescence staining of Pdx1 (G, J), Lef1 (H,K) and overlay of Pdx, Lef1 and Desmin (I, L) in E13.5 wild type pancreas (G–I) and Wnt7b PKO (J–L).

the mesenchyme to test to what extent exogenous Wnt7b promotes epithelial growth. Indeed recent *in vitro* culture of pancreatic epithelial progenitor cells indicates that the canonical Wnt agonist R-spondin indeed promotes progenitor growth (Huch et al., 2013; Jin et al., 2013; Sugiyama et al., 2013). Another possibility for the reduced pancreas in the Wnt7b overexpressing embryos could be due to negative effects of hyperactivation of canonical Wnt signaling beyond a given threshold as previously observed during endoderm patterning in *Xenopus* embryos (Zhang et al., 2013).

Our findings underscore the importance of an optimal threshold of canonical Wnt signaling for pancreatic progenitor growth and morphogenesis, as hyperactivity of this pathway disrupts morphogenesis and differentiation. Though we have identified Wnt7b to operate through the canonical Wnt signaling pathway, its effect on non-canonical Wnt signaling in the pancreas remains to be determined. Canonical and non-canonical Wnt signaling have previously been shown to be mutually antagonistic (Gerdes et al., 2007). Indeed the polycystic duct-like complexes that result from Wnt7b overexpression in the pancreas are consistent with defective non-canonical Wnt signaling. Similar polycystic ductal structures in the kidney have been associated with defective planar cell polarity, a branch of non-canonical Wnt signaling (Patel et al., 2008; Simons and Walz, 2006).

Wnt mediated epithelial-to-mesenchymal interaction in progenitor growth

It is interesting to note that cell-intrinsic abrogation of Wnt signaling either in the pancreatic epithelium or the pancreatic mesenchyme independently leads to pancreatic hypoplasia. Deletion of β-catenin exclusively in the pancreatic mesenchyme results in a similar degree of reduction in pancreatic mass as is the case for β-catenin deletion in the epithelium (Baumgartner et al., 2014; Landsman et al., 2011). This suggests that active Wnt signaling is required both in the epithelium and mesenchyme to support pancreatic progenitor cell growth. Indeed the observations in this study are consistent with the notion that Wnt7b signals in an autocrine (within the epithelium) and paracrine manner (to the mesenchyme) in the developing pancreas. The strong canonical Wnt responsiveness of the pancreatic mesenchyme (based on observed increased expression of Lef1) and the effect of Wnt7b overexpression on the mass and gene expression of the pancreatic mesenchyme suggests that epithelial-derived Wnt7b promote epithelial–mesenchymal interaction by modulating the growth and/or nature of the mesenchyme. Also, the similarity in phenotypes between the Wnt7b overexpressing pancreas (shown in this study) and the epithelial specific hyperactivation of β-catenin in the early pancreas (Heiser et al., 2006) reflects an autocrine Wnt7b signaling within the developing pancreatic epithelium. Further studies will be required to allow for a comprehensive evaluation of the functional role of Wnt7b in mediating epithelial–mesenchymal interaction during pancreatic progenitor cell growth. Future analysis focused on purifying the pancreatic epithelial and mesenchymal cell layers in the *Wnt7b* DTG will allow for detailed characterization of the effects of Wnt7b on both germ layers. Also purification and analysis of the pancreatic mesenchyme in the *Wnt7b* PKO would provide further insight into whether the proliferative effects of Wnt7b are through autocrine signaling within the epithelial layer, or via paracrine signaling through the mesenchyme, or both. It will also be interesting to evaluate a possible role of Wnt7b to recruit/promote the growth of particular mesenchymal cells to the nascent pancreatic epithelium to aid pancreatic progenitor growth.

Growth of the pancreatic epithelium has long been shown to depend on trophic factors emanating from the adjacent mesenchyme, most notable of which is Fgf10. Targeted deletion of Fgf10

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prior to the onset of pancreas development results in a severely stunted pancreatic growth. Though Fgf10 expression begins to diminish starting from E11.5 onwards, there is still an increased pancreatic progenitor cell growth beyond this stage. Our observation that Wnt7b is required for progenitor growth starting at E11.5 suggests that Wnt7b possibly operates in relay to Fgf10 to promote pancreatic progenitor growth. In such a model, Fgf10 from the mesenchyme would support the growth of the primary transition stage pancreas (~E8.5 to E12.5) following which epithelial-derived Wnt7b becomes important for subsequent progenitor cell growth. Further support for this notion can be derived from a recent *in vitro* explant study by Greggio et al. in which Fgf signaling was required for the early but not later stages of *in vitro* culture of embryonic pancreatic organoids (Greggio et al., 2013). Future studies based on the conditional deletion of Fgf10 during the secondary transition stages of pancreas development would help test whether, and to what extent Fgf10 is required for later stages of pancreatic progenitor cell growth *in vivo*.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.12.031.

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