Dominant and context-specific control of endodermal organ allocation by Ptf1a

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ABSTRACT

The timing and gene regulatory logic of organ-fate commitment from within the posterior foregut of the mammalian endoderm is largely unexplored. Transient misexpression of a presumed pancreatic-commitment transcription factor, Ptf1a, in embryonic mouse endoderm (Ptf1aEDD) dramatically expanded the pancreatic gene regulatory network within the foregut. Ptf1aEDD temporarily suppressed Sox2 broadly over the anterior endoderm. Pancreas-proximal organ territories underwent full tissue conversion. Early-stage Ptf1aEDD rapidly expanded the endogenous endodermal Pdx1-positive domain and recruited other pancreas-fate-instructive genes, thereby spatially enlarging the potential for pancreatic multipotency. Early Ptf1aEDD converted essentially the entire glandular stomach, rostral duodenum and extrahaematatic biliary system to pancreas, with formation of many endocrine cell clusters of the type found in normal islets of Langerhans. Sliding the Ptf1aEDD expression window through embryogenesis revealed differential temporal competencies for stomach-pancreas respecification. The response to later-stage Ptf1aEDD changed radically towards unipotent, acinar-restricted conversion. We provide strong evidence, beyond previous Ptf1a inactivation or misexpression experiments in frog embryos, for spatiotemporally context-dependent activity of Ptf1a as a potent gain-of-function trigger of pro-pancreatic commitment.

KEY WORDS: Pancreas, Glandular stomach, Ptf1a, Pdx1, Respecification, Mouse

INTRODUCTION

Embryonic endodermal patterning directs naïve progenitors towards regionalized competence states and subdivides them into appropriately located organ-specific progenitors. Orderly analysis of the gene regulatory logic that resolves organ-specific identities would yield crucial insight into the first steps of organogenesis. Understanding how gene regulatory networks interact with each other, presumably often in mutually repressive and auto-regulatory feed-forward loops, could provide more input into how organ territories are defined, how endoderm-mesoderm communication occurs, and how gene dysregulation leads to aberrant epithelial maintenance.

The pancreas is initiated from separate domains in the posterior dorsal and ventral foregut endoderm. The basic helix-loop-helix TF Ptf1a is expressed within endoderm throughout the pancreatic bud progenitors, subsequently within the growing tips of the branched epithelium and finally in their subsequently derived acinar cells (Krapp et al., 1998; Kawaguchi et al., 2002; Zhou et al., 2007; Masui et al., 2007). In the early multipotent progenitor cells (MPC), Ptf1a is part of a complex, PTF1, with a canonical E-protein (E12, E47 or HEB) and the nuclear mediator of Notch signaling, RBPJκ. As multipotent cells transition to acinar-lineage-restricted fates, increased expression of RPB1, a Notch-independent RBPJκ paralog, replaces RBPJκ in PTF1 (Masui et al., 2007). Thus, two types of PTF1, PTF1-J and PTF1-L, promote pro-pancreatic multipotency or acinar gene regulatory networks, respectively.

Ptf1a and its interaction with RBPJκ are necessary for pancreatic specification. Mutation of Ptf1a rendering it null or incapable of interacting with RBPJκ directs progenitors to other organ fates (Kawaguchi et al., 2002; Masui et al., 2007). The tiny pancreatic rudiment in Ptf1a−/− embryos, with endocrine and non-acinar exocrine cells, suggests that pancreatic differentiation pathways are still significantly open even without this key MPC factor. Previous work in Xenopus tested Ptf1a as a dominant inhibitor of pancreas fate (Afelik et al., 2006; Jarikji et al., 2007), but left important issues
unanswered, including the temporal and spatial windows during which such effects can be elicited, and the completeness of conversion. Particularly important issues include the duration of ectopic Ptf1a required for tissue conversion, and the effect on other genes that supposedly act together with Ptf1a as upper-level regulators of the cascade that regionally specifies pancreatic MPC and moves them to fully differentiated cells. Moreover, it is essential to understand these issues in the mammalian embryo.

We therefore addressed the potency of Ptf1a as a single TF in dominantly instructing organ fate in mammalian endoderm. Transient endodermal Ptf1a misexpression caused a coherent change in the TF expression domains in the endoderm, including broad, rapid expansion of Pdx1 expressed from its endogenous locus and recruitment of other pancreatic-progenitor identifier genes. Early transient Ptf1a stably converted pancreas-adjacent endoderm, which normally forms the glandular stomach, extra hepatic biliary system and rostral-most duodenum, into pancreatic tissue comprising its full complement of cell fates, including endocrine cells. Altering the timing of Ptf1a misexpression defined the developmental period over which dominant respecification of the endoderm became switched from early multipotent to lineage-restricted acinar transformation. We also defined a period in which endoderm and endoderm-associated mesoderm displayed interdependent specification programs.

**RESULTS**

**Transient endodermal Ptf1a misexpression**

Transient endodermal Ptf1a misexpression, or Ptf1a\(^\text{EDD}\) [endoderm], used a three-allele system: Sox17\(^\text{GFPCre}\) pan-endodermal Cre driver; ROSA26\(^\text{tTA.IRES.EGFP}\), which Cre-dependently expresses the reverse tetracycline transactivator (rtTA) and green fluorescent protein (EGFP); and tetO\(^\text{Ptf1a.IRES.lacZ}\), expressing Ptf1a and β-galactosidase (β-Gal) in the presence of rtTA and doxycycline (Fig. 1A). Single doxycycline injections produce pulsed Ptf1a expression. Sox17 is essential for endoderm formation and is expressed throughout the early definitive endoderm (Kanai-Azuma et al., 2002). Analysis of a ROSA26 reporter with an independent Sox17-Cre allele (Engert et al., 2009) indicated recombination starting as early as E7.5, with the ROSA26 promoter maintaining extensive endodermal expression until at least E16.5. Our own monitoring of EGFP expressed from ROSA26\(^\text{tTA.IRES.EGFP}\) at E10.5 and E12.5 showed recombination broadly throughout the endoderm and endothelium, with the ROSA26 promoter equivalently active during the test windows used herein (not shown). Therefore, variable response to doxycycline-induced Ptf1a\(^\text{EDD}\) cannot be attributed to shutting down of the rtTA expression system. Appropriate system behavior was also validated by post-doxycycline pan-endodermal β-Gal (e.g. Fig. 1D). Ectopic Ptf1a in trigeneric animals was only activated in endoderm and endothelium in the presence of doxycycline.

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**Fig. 1. Conditional endodermal Ptf1a misexpression (Ptf1a\(^\text{EDD}\)).** All E10.5 tissues except G.J. (A) Ptf1a\(^\text{EDD}\) system. Black triangles, loxP sites. (B,C) Morphologies of control and Ptf1a\(^\text{EDD}\) endoderm; whole-mount immunolabelling (maximum-intensity projection), pan-endodermal Foxa2. (D) tetO allele activation by β-Gal immunolabelling. (E) Control endodermal Ptf1a was in pancreatic progenitors (dorsal pancreas, yellow arrowhead; intestine, white arrowhead). (F) Ptf1a\(^\text{EDD}\) embryos showed pan-endoderm Ptf1a at E10.5, including regions normally devoid of expression (e.g. anterior stomach progenitors, white arrowhead; dorsal pancreas, yellow arrowhead). (G) 72 h (E12.5) after initiating Ptf1a\(^\text{EDD}\), Ptf1a\(^\text{+}\) tissue was more restricted, with an anterior boundary within prospective stomach (posterior stomach, yellow arrowhead; anterior stomach, white arrowhead). (H) Control E10.5 embryo, Nkx6.1/ Pdx1 co-production in dorsal pancreatic anlage. (I) With Ptf1a\(^\text{EDD}\), Nkx6.1 was suppressed in the pancreatic buds, but was restored (J) to normal levels (dorsal bud shown) by E12.5. Scale bars: B-D, 100 µm; E-J, 20 µm. dp, dorsal pancreas; int, intestine; Ig, lung; lv, liver; st, stomach; vp, ventral pancreas.
Ptf1aEDD broadly expands the Pdx1+ endodermal territory and pro-pancreatic regulatory network

We next examined alterations in early regional gene expression patterns in the foregut endoderm. Pdx1 and Ptf1a are the best-known markers linked to pancreatic MPC specification (Zhou et al., 2007; Pan et al., 2013). Pdx1 expression at E10.5 normally marks endoderm representing the dorsal and ventral pancreatic buds, caudal-most stomach, rostral duodenum and a proportion of the extra-hepatic biliary system (EHBS) (Fig. 2A). Endodermal Ptf1a is restricted to the developing pancreas within this territory. Both genes are required for proper pancreatic outgrowth and differentiation (Jonsson et al., 1994; Schaffer et al., 2010). Our observation of transient Nkx6.1 suppression (n=3) provides further indirect evidence for transient Ptf1aEDD. Analysis 24 h after E9.5 doxycycline administration showed strong Nkx6.1 suppression within pancreatic bud MPC (Fig. 1I). We speculate that endogenous Ptf1a added to Ptf1aEDD was greater than in normal pancreatic MPC, thus suppressing Nkx6.1, and that the post-doxycycline decline in Ptf1aEDD restored the Nkx6.1+ state (n=3; for 72 h post doxycycline, Fig. 1J). We propose that the post-doxycycline decline led to lower level, normally regulated Ptf1a from its endogenous locus, allowing the foregut endoderm either to restore normal pancreatic MPC specification or undergo pro-pancreatic conversion with intermediate acquisition of a pro-pancreatic state.

Ptf1aEDD induced at E9.5, analysis 24 (E10.5) or 72 (E12.5) h post-injection. (A-D) Whole-mount immunolabeling: Pdx1, Sox2. (A) Pdx1 and Sox2 induced at E9.5, analysis 24 (E10.5) or 72 (E12.5) h post-injection. (B) Pdx1+ domain greatly expanded by Ptf1aEDD. (C) Low-level Pdx1 expression was observed in tissue representing the region normally turning into stomach (Fig. 1G) and anterior duodenum. (D) Ptf1aEDD embryos, many Hnf4α+ hepatoblasts expressed low levels of Pdx1 (arrowhead). Inset: a different embryo showing a few groups of Pdx1+ cells adjacent to Hnf4α+ cells. Approximate sectional plane indicated in G-I. Scale bars: A, B, 100 µm; C, D, 50 µm; E-J, 20 µm. dp, dorsal pancreas; gb, gall bladder; int, intestine; Ig, lung; lv, liver; st, stomach; vp, ventral pancreas.
Offield et al., 1996; Holland et al., 2002; Krapp et al., 1998; Kawaguchi et al., 2002). In Ptf1aEDD embryos at E10.5, the region of Pdx1+ cells was reproducibly and greatly expanded into the more rostral endoderm and caudal ventral foregut (n=6; Fig. 2B; supplementary material Movies 1 and 2). We reproducibly observed clear suppression of Sox2 in Ptf1aEDD anterior endoderm in whole-mounts (n=5; Fig. 2B; supplementary material Fig. S1) or multiple independent side-by-side section analyses (not shown). For unknown reasons, the posterior Pdx1 expansion was limited and patchy. Despite widespread endodermal activation of ROSA26rtTA.IRES.EGFP, the tetO\(^{-}\)Ptf1a.IRES.lacz allele was poorly activated in the prospective mid-hindgut (supplementary material Fig. S2). Accordingly, Cdx2 expression and general histology were normal in the rostral-most remaining small intestine in our postnatal Ptf1aEDD analysis (not shown). This situation prevents rigorous conclusions regarding effects of Ptf1aEDD in posterior endoderm, and we therefore focus on the rostral-most duodenum and regions located rostrally and ventrally to it. Because Sox17 is also expressed within endodermal cells, it was important to address effects of endodermal Ptf1a misexpression on endodermal patterning. We misexpressed Ptf1a using the endodermal TieCre driver (Gustafsson et al., 2001) in TieCre; ROSA26rtTA.IRES.EGFP; tetOPtf1a.IRES.lacz embryos. Endothelial Ptf1a, with the same injection regimen as Sox17Cre-based Ptf1aEDD at E9.5, did not change the Pdx1 expression domain at E10.5 (n=3; supplementary material Fig. S3) or affect other regional (Nkx6.1, Pdx1) expression patterns at E12.5 (data not shown). Therefore, Ptf1a must be endodermally delivered to cause repatterning and organ conversion. We cannot, however, rule out the possibility that endothelial Ptf1a somehow supported or synergized with the Ptf1aEDD phenotype; but we note that other endodermal Cre drivers do not offer the timing and broad endodermal characteristics, are substantially mosaic or are expressed in other key tissues known as essential in patterning the embryo.

The posterior ventral foregut produces liver, EHBS (extra hepatic biliary ducts, gall bladder) and ventral pancreas (McCracken and Wells, 2012). The presumptive ventral pancreas and gall bladder are distinguishable by Sox17 expression, which becomes progressively restricted to the gallbladder anlage (Spence et al., 2009), and by Pdx1 expression. At 10.5, the ventral pancreas bud displays uniformly high Pdx1, while the gall bladder primordium shows lower, variable levels of Pdx1, which are shortly thereafter reduced further; i.e. the Pdx1+ domain seems to recede towards the ventral pancreas (Fig. 2C). With Ptf1aEDD, the Pdx1+ territory was vastly expanded ventrally, and was uniformly high within the posterior ventral foregut, suggesting that Pdx1 was ectopically expressed throughout the EHBS primordia and a substantial proportion of the nearby liver anlage (n=6 individual embryos; Fig. 2D). To verify ectopic Pdx1 activation in hepatoblasts, we tested for co-production of Pdx1 and the hepatoblast marker Hnf4a, which normally define complementary domains (n=3; Fig. 2E). Ptf1aEDD embryos showed many co-producing cells in ventral foregut endoderm (Fig. 2F). In Ptf1aEDD embryos analyzed at E12.5, no distinct gall bladder or EHBS could be found, consistent with the idea that their progenitors had been respecified and joined a common state with the ventral pancreatic bud (n=6). Serial-section analysis failed to show ectopic Pdx1+ cells at E12.5 within the distinct liver domain, indicating resolution of the early mixed pancreas/liver state as the tissue moved into liver differentiation (Fig. 2J). We cannot rule out that some Ptf1a-expressing hepatoblasts or foregut progenitors did undergo respecification and move physically into adjacent respecified domains. We addressed a potential cell-death explanation below. The cell migration issue would require early-specification-marker-driven and temporally specific lineage tracing by non-Cre methods (because we used Cre to activate Ptf1aEDD), which are currently unavailable.

We tested whether Pdx1+Ptf1a+ endodermal progenitors induced by Ptf1aEDD expressed other pancreatic MPC markers. In normal E10.5-12.5 endoderm, Nkx6.1 specifically marks pancreatic progenitors (Pedersen et al., 2005), and therefore was not expressed in control Pdx1+ stomach or duodenum (not shown). Analyzing Ptf1aEDD embryos at E12.5, we observed co-production of Nkx6.1 within the Pdx1+Ptf1a+ presumptive posterior stomach (n=6; Fig. 2G). We reproducibly detected similar expansion of Prox1, another influential pancreatic progenitor TF not normally expressed in the prospective stomach (n=3; Fig. 2H). Nkx6.1 was also ectopically expressed in the domain that would normally produce anterior duodenum (Fig. 2I). We examined whether proliferative expansion of pancreatic progenitors contributed to the spatial expansion of pancreatic markers in Ptf1aEDD embryos. We presume that replacement of stomach progenitors by hyper-proliferative pancreatic progenitors would also involve loss of progenitors for other organ territories. In Ptf1aEDD embryos at E10.5, we were unable in this analysis to use pancreas-specific markers such as Ptf1a, which was expanded throughout the endoderm, or Nkx6.1, which was temporarily suppressed. Given that the vast expansion of Pdx1 occurs acutely following Ptf1aEDD, we examined E10.5 Ptf1aEDD embryos by extensive sectional analysis for regional alterations in proliferation using phospho-histone H3 (chosen over e.g. Ki67 or BrdU labeling, because of the high proliferation rate of cells in all tissues over these stages of organogenesis). We also tested for increased apoptosis (cleaved caspase-3) within the Pdx1+ domain. We detected no regional alterations in proliferation or apoptosis that could explain the expansion in the Pdx1+ domain as spreading of cells from the normal pancreatic domain (not shown).

These combined alterations in progenitor markers suggest that Ptf1aEDD initiated a dominant effect on the posterior foregut, initially activating expression of Pdx1, a principal component of the pro-pancreatic MPC gene regulatory network, from its endogenous locus and much more widespread than normal. The expression of other pro-pancreatic MPC regulatory-network members was recruited to the remaining Ptf1a+Pdx1+ region (more rostral regions withdrawing from this condition), stabilizing a pro-pancreatic condition in what would normally have become the prospective glandular stomach, anterior duodenum and EHBS.

**Respecified pancreas-adjacent endoderm produces differentiated pancreatic cell types**

To test whether Ptf1aEDD initiated a complete conversion to pancreatic tissue, misexpression was initiated at E9.5 and tissues analyzed at E18.5/P0 when differentiation programs are clearly distinguishable. Whereas some aspects of pancreas differentiation are discernible mid-gestation, the programs in stomach and intestine acquire many cell-type-specific characteristics only at later gestation and peri-/postnatally. Ptf1aEDD pups were stillborn (probably caused by deficits in endothelial tissues), but otherwise equivalent in size to the various controls described above.

The gross anatomy of foregut endodermal organs (see e.g. supplementary material Fig. S4 for lung) was normal except in the posterior foregut (described below), and cellular differentiation was largely normal in the lung and forestomach in Ptf1aEDD pups. Because Ptf1aEDD pups were stillborn, their lungs did not inflate, but there was appropriate differentiation of proximal and distal lung, and extensive endothelial coverage of terminal airways (supplementary material Fig. S4). The Ptf1aEDD forestomach contained multiple layers of Sox2+ cells (supplementary material Fig. S5). These data indicate that, despite ectopic Ptf1a expression and effects on Sox2, the lung resisted...
Ptf1a-mediated respecification and the normal differentiation program was reassessed. Although the lack of forestomach-specific markers limited our depth of analysis, we conclude that the forestomach and lung were not grossly diverted from their normal organ identities, albeit suffering possible modest alterations in terminal differentiation.

Abnormalities in embryos/pups from E9.5 Ptf1aEDD (n=6) included disrupted gastrointestinal tract continuity at the stomach-duodenum junction, an abnormal shape and color of the stomach (Fig. 3B,D), and lack of a morphologically obvious EHBS (Fig. 3C,E). Pancreatic acini, the predominant pancreatic cell type, are visually distinguishable by their opacity and characteristic cauliflower-like tissue structure. The posterior stomach in Ptf1aEDD pups contained tissue externally resembling pancreatic acinar cells (Fig. 3B,D). The morphology, size and position of the dorsal pancreas were visually normal. The ventral pancreas resided near the break between the posterior stomach and blind end of the rostral-most duodenum (Fig. 5D).

The postnatal and adult mouse stomach comprises three distinct domains (Lee et al., 1982). The most rostral forestomach is overall typified by a pseudo-stratified keratinized epithelium. Caudal to the forestomach lies the glandular stomach, divided into the corpus and antrum. Both domains have columnar epithelial morphology (Fig. 3F) but different cell types. Parietal and chief cells are derived only from the corpus, whereas the antrum contains mucus-secreting cells. Essentially replacing the entire glandular stomach epithelium in Ptf1aEDD pups were cells indistinguishable from pancreatic acini by morphology and H&E staining (large eosinophilic cells) (Fig. 3G,H). It appeared as if pancreatic tissue had replaced the entire glandular stomach (Fig. 3I-J).

In normal animals, the mesoderm surrounding the stomach (Fig. 3F) is distinct from the mesoderm at the pancreas. Stomach-associated mesoderm becomes multi-layered, including submucosa and muscularis layers between the coelomic and endodermal epithelia. This normal stomach-associated mesoderm was absent next to the ectopic pancreas in the posterior stomach of Ptf1aEDD pups. The ectopic pancreas was closely apposed to coelomic epithelium, and the mesoderm was characteristically pancreatic, with pervasive interdigitation among the ectopic differentiated epithelial tissue (Fig. 3H). These data support the conclusion that, following early conversion of the endoderm that would have formed the prospective stomach, anterior duodenum and EHBS into pancreatic MPC, there was region-appropriate dominant instruction of the overlying mesoderm cell fate.

The visual lack of EHBS or anterior duodenum was corroborated by systematic serial-section analysis of Ptf1aEDD tissue, which found no evidence for distal EHBS tissue types (gall bladder, cystic duct) or structures of the anterior duodenum, including the pyloric connection to stomach or the Brunner’s glands normally found at the rostral-most collar region of the duodenum. Ptf1aEDD tissue contained some large DBA-reactive ducts adjacent to the liver and somewhat posterior to the pancreas-converted stomach, which could have represented a fragment or partially formed segment of the normal proximal EHBS (supplementary material Fig. S5). Small patches of pancreas tissue were found between the coelomic epithelium and submucosa along a limited proportion of the small intestine (supplementary material Fig. S5). From this morphological and histological evidence, we infer that transient Ptf1aEDD at E9.5 caused glandular stomach, anterior duodenum and distal EHBS progenitors to become developmentally respecified into pancreas. The fate switch of gut-tube endodermal progenitors, and the change in character of the associated adjacent mesoderm, led to a break in stomach-intestine continuity and loss of a complete EHBS.

**Fig. 3. Pancreatic cells replacing glandular stomach, anterior duodenum, EHBS.** (A) Set-up: E9.5 doxycycline transiently pulsed ectopic Ptf1aEDD, analysis at E18.5/P0. (B,C) Morphology of control stomach, duodenum (B), extra-hepatic biliary system (EHBS; C). Closed and open arrowheads in C: cystic duct, gall bladder. (D) With Ptf1aEDD, pancreatic acinar cells found within posterior stomach. Stomach and intestine were disconnected (yellow arrowheads). (E) Distal EHBS was absent. (F) H&E staining, control glandular stomach. Dashed lines: submucosa (red), muscularis layers (yellow). (G) H&E-stained control pancreas. (H) Glandular stomach in Ptf1aEDD embryos replaced with pancreatic acini. The thick mesenchymal layer normally surrounding glandular stomach was absent adjacent to ectopic pancreas (arrowheads). (I) Close-up from G. (J) Additional image, Ptf1aEDD glandular stomach. Scale bars: B-E, 1 mm; F,G,I,J, 50 µm; H, 500 µm. sp, spleen; duo, duodenum; lu, stomach lumen; m, mucosa; a, acini; i, islet; fs, forestomach.

**Ectopic pancreas tissue replacing the glandular stomach contains endocrine cell types**

We focused in the next analysis on the stomach-associated ectopic pancreas, because we were unable to distinguish between the true ventral pancreas and pancreas tissue that had become continuous with it but was derived from the Ptf1aEDD-converted EHBS or rostral
duodenum. To determine the extent of pancreas conversion, we
serially sectioned the entire foregut from three Ptf1aEDD pups, using
molecular markers to identify gastric and pancreatic cell types on a
majority of these sections to ensure detailed tissue coverage. Parietal
cells are restricted to glands of the corpus and are marked by high HK-
ATPase, whereas epithelium of the corpus and antrum is generally
marked by GSII lectin (Griffonia simplicifolia Lectin II). Thus, GSII/
HK-ATPase double-positive glands identify corpus (Fig. 4A) and
GSII+/HK-ATPase− glands signify antrum. Ptf1aEDD−converted
posterior stomach contained only minuscule, scattered GSII+/HK-
ATPase− or GSII+/HK-ATPase− areas. Fig. 4B deliberately shows one
of those remaining areas, with the overwhelming majority of cells
within the posterior stomach expressing pancreatic acinar-specific
amylase (Fig. 4B).

Pancreatic endocrine and duct cells were present within the ectopic
pancreas. Pancreatic endocrine cells are generally synaptophysin+,
whereas duct cells are DBA-lectin-reactive (Fig. 4C). Epithelial
endocrine cells in the stomach normally distribute as single cells,
whereas most endocrine cells in the pancreas cluster into the often
large islets of Langerhans, with insulin signifying pancreatic identity
(Fig. 4E). Consistent with the idea that early, transient Ptf1aEDD did
not deflect normal pancreatic organogenesis in a major way,
immunodetection and histological examination showed that islet-
cell representations and clustering in the ‘endogenous pancreas’
after E9.5 Ptf1aEDD were equivalent to normal controls (data not
shown). Within the ectopic pancreas replacing the glandular
stomach, we found synaptophysin+ cells, both clustered and
dispersed, and DBA+ duct cells. Dispersed endocrine cells were
much more numerous than those organized into scattered, loosely
organized clusters (typified by Fig. 4D), but were reproducibly
present throughout the ectopic pancreas (see also supplementary
material Fig. S6). All endocrine cells were single-hormone positive
and expressed insulin, glucagon or somatostatin (Fig. 4F). Insulin+ cells
within the ectopic pancreas expressed Pdx1 and Nkx6.1; criteria for equivalence to normal β-cells (Fig. 4G,H). These cells are
too few for isolation and physiological testing of their maturity
and glucose responsiveness. The morphological, histological and
molecular analysis supports the conclusion that transient Ptf1aEDD
at E9.5 caused functional respecification of the prospective stomach
into multipotent pancreatic progenitors that were then capable of
deriving all pancreatic lineages. Possibly, the Ptf1aEDD-induced
conversion from an epithelial naïve gut tube to bud-like pre-
pancreatic tissue (from section analysis of E12.5 embryos; data not
shown) somehow constrained the ability to form and organize
endocrine cells within the ectopic pancreas.

Certain non-pancreatic progenitors maintain competence for Ptf1a-mediated respecification

Given the striking re-specification of glandular stomach, anterior
duodenum and EHBS, we tested whether their competence to
respond to Ptf1aEDD became more restricted over developmental
time. Tissue gathered after doxycycline injection at E10.5 (n=6) or
E11.5 (n=7) was similar to the E9.5 Ptf1aEDD situation, with
pancreatic acinar cells located throughout the glandular stomach.
The rostral-most end of the small intestine seemed closer to the
posterior stomach compared with E9.5 induction, suggesting loss of
less intestinal tissue, but the stomach-intestine connection was still
interrupted by pancreas tissue (Fig. 5B,D). The distal EHBS was
still absent under E10.5 or E11.5 Ptf1aEDD (Fig. 5C,E).

Ptf1aEDD initiated at E12.5 (n=5) still caused substantive
replacement of the majority of the glandular stomach with
pancreatic acini (Fig. 5F). This stage of Ptf1aEDD was the first at
which the continuity of the gastrointestinal tract at the stomach-
intestine junction was no longer interrupted and the distal EHBS
was morphologically apparent (Fig. 5G). Sectional analysis
revealed that the entire EHBS was replaced with pancreatic acini
surrounding a central duct (Fig. 5H). Although the overall gross
morphology of the intestine appeared normal, serial sectioning of
Ptf1aEDD tissue revealed a few small patches of pancreatic acinar
cells scattered within the anterior duodenum (Fig. 5I).

We compared the morphological and molecular responses to Ptf1aEDD initiated at E10.5, E11.5 or E12.5, with reference to the
E9.5 data, by thorough serial-section analysis of three pups per
stage. The extent of pancreas replacement within the glandular stomach with Ptf1a\textsuperscript{EDD} instigated at E10.5 or 11.5 was almost equivalent to the E9.5 Ptf1a\textsuperscript{EDD} effect. In the limited regions not showing pancreas conversion, the normal thickened glandular stomach mesenchyme was present, but was otherwise pancreas-type elsewhere (Fig. 6A,B). After Ptf1a\textsuperscript{EDD} at E12.5, replacement of the glandular stomach with pancreatic acini was substantial, but becoming much more focal. There was a universal maintenance of stomach mesenchymal character with Ptf1a\textsuperscript{EDD} at any point from E12.5 onward (Fig. 6C,D).

Formation of pancreatic acinar tissue following Ptf1a\textsuperscript{EDD} at E10.5, E11.5 or E12.5 occurred throughout the glandular stomach, including the corpus (amylase expression interspersed with GSII+/HK-ATPase+ glands) (Fig. 6E,F). After Ptf1a\textsuperscript{EDD} at E10.5 or E11.5, insulin\textsuperscript+ cells within the ectopic pancreas tissue in the glandular stomach were rare and were not found in clusters (Fig. 6G). No insulin\textsuperscript+ cells were detected within the converted stomach, with Ptf1a\textsuperscript{EDD} initiated at E12.5 (Fig. 6H) or thereafter.

In summary, the endodermal progenitors of the glandular stomach (corpus, antrum), anterior duodenum and EHBS retained the ability, until at least E12.5, to convert to pancreas tissue under Ptf1a\textsuperscript{EDD} challenge. Between E9.5 and E12.5, the conversion transitioned from complete (whole pancreas) tissue-level replacement to progressively more focal and single-lineage (acinar) cell types. The completeness of pancreatic respecification with E9.5 Ptf1a\textsuperscript{EDD}, as scored by all

Fig. 5. Posterior foregut anatomical alterations after shifting the Ptf1a\textsuperscript{EDD} window. (A) Ptf1a\textsuperscript{EDD} induced at E10.5, E11.5 or E12.5, and analyzed at E18.5/P0. (B,C) Morphology of stomach, duodenum (B) and EHBS (C) after E10.5 Ptf1a\textsuperscript{EDD}. (B) Posterior stomach replaced by pancreas tissue, with disconnected blind-ended intestinal tube. Arrowheads: pancreatic tissue present in this location. (C) Definitive EHBS structures were absent (arrowhead and data not shown). (D,E) Similar alterations with E11.5 Ptf1a\textsuperscript{EDD}. (E) Tissue from a separate animal, focused on continued lack of EHBS, but presence (arrowhead; close-up in inset) of several small patches of acinar clusters. (F-I) E12.5 Ptf1a\textsuperscript{EDD}. (F) Pancreatic tissue still replaced much of the glandular stomach (arrowhead); definitive acini are shown in Fig. 6. Stomach and duodenum were now connected (yellow line). (G) EHBS structures were apparent, but showed conversion of most of its endodermal epithelium to acini. Insets: close-ups of both ends, centered on the two yellow arrowheads. Right panel, tissue at the intestinal junction (asterisk). (H) Histology showed this EHBS to comprise pancreatic acini surrounding a central duct. (I) Pancreatic cells were also scattered within intestinal tissue (arrowhead). Scale bars: B-G, 1 mm; H,I, 50 µm. lv, liver.

Fig. 6. Temporal competence of Ptf1a\textsuperscript{EDD} respecification. Ptf1a\textsuperscript{EDD} at E10.5, E11.5 or E12.5; analysis at E18.5/P0. (A,B) Posterior stomach with E10.5 (A) or E11.5 (B) Ptf1a\textsuperscript{EDD}. Pancreatic acinar-like cells replaced the columnar epithelium. Normal stomach-type mesenchyme was absent adjacent to ectopic pancreas (arrowheads). (C,D) E12.5 Ptf1a\textsuperscript{EDD}; pancreatic acini still largely replaced glandular stomach epithelium (C,D: anterior, posterior stomach), but stomach mesenchyme had its normal phenotype adjacent to ectopic pancreas. With (E) E10.5 or (F) E12.5 Ptf1a\textsuperscript{EDD}, pancreatic acini were found next to HK-ATPase\textsuperscript+ corpus glands. (G) With E11.5 Ptf1a\textsuperscript{EDD}, pancreatic endocrine cells in converted tissue were unclustered and much less prevalent than with E9.5 Ptf1a\textsuperscript{EDD}. Very few insulin\textsuperscript+ cells were detected. (H) E12.5 Ptf1a\textsuperscript{EDD}, no insulin\textsuperscript+ cells were detected. Single, dispersed, synaptophysin\textsuperscript+ cells were detected, but normal stomach also contains endocrine cells. Extensive analysis of Ptf1a\textsuperscript{EDD} stomach showed transformations were to pancreatic acini. Scale bars: A-C, 250 µm; D, 100 µm; E-H, 20 µm.
pancreatic lineages, including insulin+ endocrine cells, diminished significantly by E10.5 and E11.5. By E12.5, respecification was less robust than earlier Ptf1aEDD and was wholly acinar-like. With Ptf1aEDD between E9.5 and E11.5, the mesoderm over the glandular stomach, anterior duodenum and EHBS apparently received instruction from the respecified endoderm to adopt a pancreatic mesodermal character. By E12.5, the fate of the overlying mesoderm in the intestine and glandular stomach was fixed in its original state.

**DISCUSSION**

Regional susceptibility to dominant Ptf1a-based endodermal conversion to pancreas

TFs can control cell-lineage decisions, dominantly reprogram differentiated cells to pluripotency or cause direct reprogramming between differentiated cell types. Our work on the organ-switching potential of Ptf1a begins to address the competitive interplay between the transcriptional regulatory networks that pattern the early mammalian posterior foregut endoderm. Under the classical specification-commitment-differentiation paradigm, we focused on the issue of whether, how and when endodermal regions become ‘irrevocably allocated’ to specific organ fates. Our study tested whether the endoderm has regional variation in the openness to transcription factor-induced fate switching or variable response over time, as the organ anlagen begin to build their internal tissue-specific gene regulatory networks. The previous studies on endoderm were mostly focused on how TFs and extracellular signals interact to control differentiation within a particular gut tube-derived organ, with pioneering work relevant to our studies centered on the decision of posterior foregut endoderm to become liver or pancreas (e.g. see Wandzioch and Zaret, 2009).

Previous studies in frog embryos (Afeilik et al., 2006; Jarijki et al., 2007) suggested that Ptf1a can divert early endoderm within the Pdx1 (XIIbox8 in frogs) territory towards pancreas fate, but our study takes crucial further steps: (1) The doxycycline-inducible system expresses unmodified Ptf1a. (2) We find early-stage endoderm competent to switch organ fates completely, forming all pancreatic lineages. There is a competence switch to lineage-restricted acinar transformations at later stages. (3) We define the responsive tissues in more detail: responsive progenitor domains include those outside the endogenous Pdx1 expression domain (e.g. not only stomach antrum but also corpus and the entire EHBS). (4) The differential sensitivity of mammalian tissue to the correct type of Ptf1a-based switching stimulus brings the endoderm-patterning closer to human relevance. (5) We show clearly that Ptf1a triggers expression of principal early-acting (‘first tier’) members of the pro-pancreatic gene regulatory network, from their endogenous loci and in particular the rapid and broad activation of Pdx1 expression, including far outside of its normal territory. (6) We study the organ-conversion process in terms of early-stage alterations in the regionalized expression of other transcriptional regulatory proteins, as well as the final cell/tissue differentiation phenotype, leading to our conclusion that certain tissues are resistant to conversion, despite the broad activation of Pdx1 expression – which in a few embryos extended even up into the vicinity of the lung bud (n=2; not shown).

Our system showed differential effects after transiently expressing this fate-triggering factor at various stages of development. Temporal changes in the progenitor response to the same factor help inform on the dynamics of epithelial competence during embryogenesis. For example, inducing Ngn3 activity at different stages of pancreas development caused changes in endocrine cell-fate choice (Johansson et al., 2007). Moreover, altering BMP and TGFβ signaling in the posterior foregut produced different effects on pancreas specification over time (Wandzioch and Zaret, 2009). An important component in our system was the transient Ptf1aEDD; too much ectopic Ptf1a, or for too long, caused early embryo death, probably from unresolvable tissue confusion or dysfunction. The transient expression allows exogenous Ptf1a to act on endogenous loci during a narrow developmental window, and we infer that the absence of continued exogenous Ptf1a allowed proper sequential self-assembly of gene regulatory networks from the endogenous genes. Endoderm farther from the pancreas, such as the prospective forestomach-esophagus and liver, also displayed Ptf1a-induced ectopic Pdx1 production. But, in the farthest reaches, the expanded Ptf1a/Pdx1 co-positive territory subsequently underwent substantial retreat (most likely by extinguishing expression, not by large-scale cell migration or apoptosis), with restoration of the original organogenesis programs of those territories.

**Endodermal progenitor competence**

The potency of Ptf1a to induce rapidly a much broader domain of expression of Pdx1 from its endogenous locus indicates a tight interdependence between these two genes. Both genes represent primary (‘first tier’) regulators of pro-pancreatic MPC character (Burlison et al., 2008; Kawaguchi et al., 2002; Krapp et al., 1998; Jonsson et al., 1994; Offield et al., 1996; Thompson et al., 2012). We speculate that acquiring a stable Ptf1a/Pdx1+ co-positive state, at a particular stage of organ development, is an efficient feed-forward method of specifying and then committing to the pancreatic fate. A pairwise threshold-dependent activation switch would be an attractive way of gating entry to specific organ commitment states. Such a relationship could explain the ability of Nkx2.1 and Pax8 by ectopic induction (although less transient than in our case) to stimulate formation of apparently genuine thyroid follicles in vitro from mouse ES cells (Antonica et al., 2012). The rapid activation of Pdx1 by Ptf1a induction and generation of a feed-forward loop is supported by evidence for Ptf1a and Pdx1 inter- and auto-regulation (Gerrish et al., 2001, 2004; Marshak et al., 2000; Masui et al., 2007; Oliver-Krasinski et al., 2009; Wiebe et al., 2007). Possibly, activation of endogenous Ptf1a involves its auto-regulatory enhancer (ARE) (Masui et al., 2008), and after activation the production of endogenous Ptf1a should help keep the Ptf1a locus active. Potentially, the ARE is accessible to exogenous Ptf1a even at late stages, as cells in the prospective glandular stomach – perhaps epigenetically and functionally related to pancreatic acinar cells – retained competence at E15.5 to initiate focal, acinar-only differentiation (data not shown). The direct downstream target genes of Ptf1a causing respecification of stomach, duodenum and EHBS, but not other endodermal regions, are unclear. Our working model, however, is that Ptf1a/Pdx1 establish a ‘first tier’ trigger of the pancreatic GRN, and that in tissues that are competent to fully stabilize their auto- and cross-regulatory interactions, they then dominantly subvert other regional organ programs. This competency state includes the ability to utilize the ‘second tier’ pro-pancreatic genes (Nkx6.1, Mnx1, Prox1 and so forth).

A side-issue relates to the effects of early Ptf1aEDD on the endogenous pancreas. The transient suppression of Nkx6.1 by Ptf1aEDD, an incorrect state with respect to normal pancreatic bud MPC, was reversed by E12.5, probably related to post-doxycycline reduction of exogenous Ptf1a. After cells exited from this transitional state, endogenous pancreas differentiation proceeded essentially normally. Later-stage Ptf1aEDD, however, appeared to promote acinar cell fates over endocrine and ductal cell fates; we propose via suppressing epithelial Nkx6.1 and Sox9, as reported by
Schaffer et al. (2010). Future studies are aimed at exploring whether the acute effects of Ptf1a misexpression in the endogenous pancreas change over time.

In resistant progenitor populations, such as the more distant anterior endoderm, Ptf1aEDD seems unable to initiate or sustain the expression from endogenous Ptf1a and Pdx1, perhaps with some threshold dependency. We are now also preparing to test genetically the possibility that the Ptf1a-Pdx1 inter-regulation, and the recruitment of second- and third-tier pro-pancreatic instructors, is countermanded by competing gene regulatory programs such as those run by Sox2. After an initial phase of suppressed Sox2 levels throughout Ptf1aEDD-by competing gene regulatory programs such as those run by Sox2.

second- and third-tier pro-pancreatic instructors, is countermanded possibility that the Ptf1a-Pdx1 inter-regulation, and the recruitment of dependency. We are now also preparing to test genetically the

It remains to be seen whether such pinpoint precision is forthcoming (M.A.H. and R.J.M., unpublished). Doxycycline (5 mg/ml) was given intraperitoneally (10 µg/g). Mouse strains, doxycycline treatments Experiments were performed in accordance with institutional and national guidelines for the care and use of laboratory animals. Sox17creIRES.EGFP (Sox17creIRES.EGFP), Tg(Tie1-Cre)9Ref (Tie1cre) and Grl(Rosa26rtTA.IRES.EGFP) were described (Choi et al., 2012; Gustafsson et al., 2001; Belteki et al., 2005). A description of jetO-Ptf1a is forthcoming (M.A.H. and R.J.M., unpublished). Doxycycline (5 mg/ml in water) was given intraperitoneally (10 µg/g).

Histology, immunofluorescence

Embryonic/perinatal tissues were fixed (4% paraformaldehyde, 4°C) for 1-4 h, or longer for older stages. Paraffin sectioning and hematoxylin & eosin (H&E) staining were described (Kawaguchi et al., 2002). For cryosection immunofluorescence, tissues were washed in ice-cold 1× PBS after fixation, sucrose-equilibrated (30%, 4°C, overnight) and OCT-embedded. Cryosections were PBS-washed, permeabilized (0.2% Triton X-100 in PBS) and blocked (1 h, 5% serum in PBS; serum species varying by secondary antibody). Primary incubation in blocking solution was overnight at 4°C. Secondary antibodies were in PBS (1 h, room temperature). Supplementary material Table S1 lists antibodies.

Whole-mount immunofluorescence

Whole embryos were fixed, gut and associated tissues were dissected out, and embryos were blocked (2 h; 1× PBS with 0.5% Tween-20, 20% serum). Primary or secondary antibody incubations were overnight (4°C; PBS with 0.5% Tween-20, 10% serum). Tissue was cleared in BABB (benzyl alcohol/ benzoic acid) for imaging.

Imaging, whole-mount rendering

LSM 510 (Zeiss) or FV-1000 (Olympus) confocal microscopes, with LSM Image Browser (Zeiss) or Axiovision 4.8 software (Olympus) produced maximum-intensity projections of z-stacks via ImageJ (NIH). Noise reduction in Photoshop solely removed the typical punctate background obscuring real signal.
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Competing interests
The authors declare no competing financial interests.

Author contributions
S.G.W. and C.V.E.W. designed the experiments, interpreted data and wrote the manuscript. M.A.H., A.G.-B., M.A.M. and R.J.M. provided essential mice and critically read the manuscript.

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Supplementary information
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References


