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The Parahox gene *Pdx1* is required to maintain positional identity in the adult foregut

ANDREW M. HOLLAND*[,1], SONIA GARCIA[1], GAETANO NASELLI[2], RAYMOND J. MACDONALD[3] and LEONARD C. HARRISON[2]

[1]Monash Immunology and Stem Cell Laboratories, Monash University, Clayton, Victoria Australia, [2]Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia and [3]University of Texas Southwestern Medical Center, Dallas, TX, USA

ABSTRACT The homeobox gene *Pdx1* is a key regulator of pancreas and foregut development. Loss of *Pdx1* expression results in pancreas agenesis and impaired development of the gastro-duodenal domain including Brunner's glands. We previously demonstrated a key role for Pdx1 in maintaining the integrity and function of insulin-secreting β cells in the adult pancreas. In the present study, we aimed to determine if expression of *Pdx1* is required to maintain the cellular identity of the gastro-duodenal domain in adult mice. Immunohistological studies were performed in a mouse model in which expression of *Pdx1* was conditionally repressed with the doxycycline-responsive tetracycline transactivator system. Mice in which *Pdx1* was chronically repressed developed hamartomas in the gastro-duodenal domain. These lesions appeared to arise from ectopic foci of anteriorized cells, consistent with a localised anterior homeotic shift. They emerge with the intercalation of tissue between the anteriorized and normal domains and appear strikingly similar to lesions in the colon of mice heterozygous for another Parahox gene, Cdx2. Continuing expression of Pdx1 into adult life is required to maintain regional cellular identity in the adult foregut, specifically at the gastro-duodenal boundary. Loss of *Pdx1* expression leads to anterior transformation and intercalary regeneration of ectopic tissue. We propose a model in which the posterior dominance of classical *Hox* genes is mirrored by the *Parahox* genes, providing further evidence of the functional conservation of the *Parahox* genes. These findings may have implications for further understanding the molecular basis of gastro-duodenal metaplasia and gastro-intestinal transformations such as Barrett's esophagus.

KEY WORDS: *Pdx1, Cdx2, parahox, Brunner's gland, tTA*

Introduction

Pdx1 is a member of the Parahox gene cluster, a paralogue of the archetypal Hox cluster thought to have arisen from the duplication of a common ancestral Protohox cluster (Brooke *et al.*, 1998; Ferrier *et al.*, 2005; Garcia-Fernàndez, 2005). The Parahox gene cluster (comprising *Pdx1*, *Cdx* and *Gsx* in mammals) is characterised largely on the basis of its phylogenetic origins and genomic organization, but recent studies have revealed conservation of other Hox-like properties for the Parahox genes (Sherwood *et al.*, 2009). Classical Hox genes are defined by several fundamental properties, including the ability to determine positional identity (homeosis), spatial co-linearity and posterior dominance of expression (Twyman, 2001). The Parahox genes Pdx1 and Cdx2 are predominantly expressed in endodermal derivatives and exhibit the classic spatial co-linearity of expression characteristic of the Hox genes (Garcia-Fernàndez, 2005). The overlapping expression profiles of the *Pdx1* and *Cdx* genes in their respective gut domains resemble those of the Hox genes, which typically exhibit strong, sharp expression boundaries anteriorly, tapering off posteriorly. Both Pdx1 and Cdx2 are known to play important and evolutionarily conserved roles in the specification or maintenance of positional boundaries in the gut (Cole *et al.*, 2009; Gaunt *et al.*, 2008; Grapin-Botton *et al.*, 2001; Zorn and Wells, 2009).

Abbreviations used in this paper: SSE, stratified squamous epithelia; tTA, tetracycline transactivator.

***Address correspondence to:** Andrew M. Holland. Monash Immunology and Stem Cell Laboratories, Level 3, Building 75, Monash University, Clayton, Victoria 3800 Australia. Tel: +61-3-9905-0654. Fax: +61-3-9905-0680. e-mail: AMHolland35@gmail.com

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Evidence of a homeotic role for Cdx2 has been deduced from the phenotype of Cdx2 heterozygous mice, which exhibit skeletal defects consistent with an anterior homeotic shift and hamartomatous polyps that arise from ectopic foci of gastric tissue in the colon (Beck *et al.*, 1999; Chawengsaksophak *et al.*, 1997; Tamai *et al.*, 1999). This role is further supported by experiments in which ectopic expression of Cdx2 in the gastric epithelium leads to intestinal (posterior) transformation while conditional inactivation of *Cdx2* in the hindgut results in anterior transformation of the posterior gut (Gao *et al.*, 2009; Grainger *et al.*, 2010). Similarly, ectopic expression of Pdx1 outside its native domain in the embryonic foregut endoderm represses genes specifically expressed anteriorly (*Sox2*) or posteriorly (*Cdx2*) (Grapin-Botton *et al.*, 2001; Silberg *et al.*, 2002).

Pdx1 is a critical regulator of pancreas development and function. It is expressed initially in the foregut endoderm at the sites from which the dorsal and ventral pancreatic buds emerge. Its expression domain then expands along the gut tube from the distal stomach through the duodenum. In *Pdx1* null mice, the pancreas fails to develop. In addition, development of Brunner's glands (mucus secreting glands located in a collar around the proximal duodenum), the major duodenal papilla and the bile duct is impaired, and the number of enteroendocrine cells is reduced (Fukuda *et al.*, 2006; Offield *et al.*, 1996). We have previously described a mutant mouse model in which expression of *Pdx1* could be conditionally repressed with the doxycycline-responsive tTA system (Hale *et al.*, 2005; Holland *et al.*, 2005; Holland *et al.*, 2002). Repressing *Pdx1* progressively throughout embryonic life demonstrated its requirement at distinct stages of pancreas development, while repressing *Pdx1* in adult mice revealed its requirement to maintain the function and allow regeneration of pancreatic β cells (Hale *et al.*, 2005; Holland *et al.*, 2005). We have now used this model to investigate the role of Pdx1 in the adult foregut.

Results

Conditional knockout of Pdx1

As *Pdx1* has been shown to be required for the correct development and specification of Brunner's glands and enteroendocrine cells in the gastro-duodenal domain of the embryonic foregut, we examined these areas in adult mice in which *Pdx1* was repressed to determine if expression of Pdx1 was required to maintain positional identity in the adult gut (Offield *et al.*, 1996).

Initially, we examined untreated $Pdx1^{tTA/tTA};Tg^{Pdx1}$ mice[18,19] to confirm that their gastro-duodenal development was normal and to identify the expression pattern of Pdx1 in the adult gut. Gross morphological analysis of $Pdx1^{tTA/tTA};Tg^{Pdx1\text{-}LacZ}$ mice expressing a LacZ reporter gene confirmed normal development of the stomach, pylorus and duodenum (Fig. 1). The strongest extra-pancreatic expression of Pdx1/LacZ was observed in a collar around the rostral duodenum and gastric pylorus, consistent with expression in the submucosal Brunner's glands (Fig. 1 A-D). Weaker more discrete expression was observed in the glandular distal stomach and proximate to the Ampulla of Vater (Fig. 1 A,C). Histological examination revealed Pdx1 expression in the pyloric and gastric glands in the distal stomach (gastric antrum) (Fig. 2A), intestinal villi and transit amplifying region of the crypts (Fig. 2B), Brunner's glands (Fig. 2C) and proximate to the major duodenal papilla where the pancreatic duct enters the duodenum (Fig. 2D), consistent with observations in earlier studies (Fukuda *et al.*, 2006; Offield *et al.*, 1996). Pdx1 expression in Brunner's glands was strong overall, with some acinar cells exhibiting markedly higher levels of expression. Expression was also strong in crypts and at the base of villi, consistent with Pdx1 expression in duodenal progenitor cells during embryogenesis and with the suggestion that Pdx1-positive crypt cells represent a subset of multipotent intestinal progenitor cells in postnatal life (Kawaguchi *et al.*, 2002; Stanger *et al.*, 2005) (Fig. 2). Following 14 days treatment with

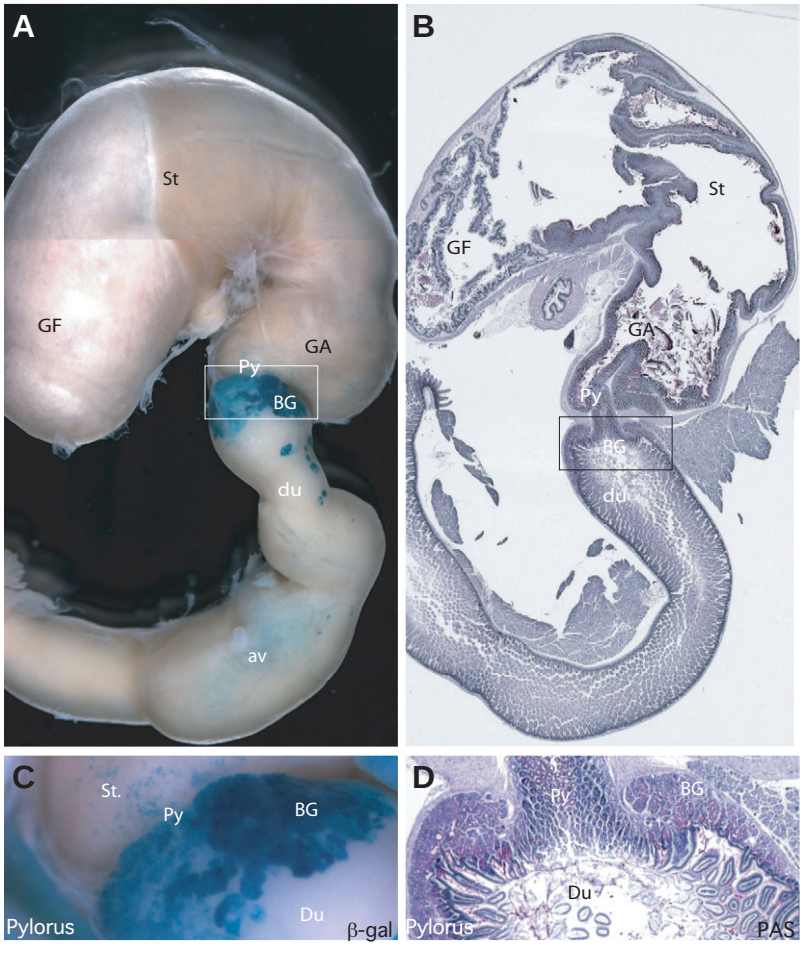

Fig. 1. Expression of Pdx1 in the adult gastro-duodenal domain. *Gastrointestinal tracts of* $Pdx1^{tTA/tTA};Tg^{Pdx1\text{-}LacZ}$ *mice.* **(A,C)** *β-galactosidase staining reveals Pdx1 expression in the distal stomach, Brunner's glands and proximate to the major duodenal papilla.* **(B,D)** *Periodic Acid Schiff stained sagittal section shows normal development of the foregut and delineation of the Brunner's glands in the* $Pdx^{tTA/tTA};Tg^{Pdx1}$ *mice.* **C** *Detail of the area delineated by the white box in* **(A)**. *shows Pdx1/LacZ expression in Brunner's gland acini and in discrete cells in the distal stomach.* **(D)** *Detail of the area delineated by the black box in* **(B)**. *shows normal development and organization of the pylorus and Brunner's gland domains. GF, gastric fundus; GA, gastric antrum; Py, pylorus; Du, duodenum; BG, Brunner's glands; St, stomach; av, ampulla of vater.*

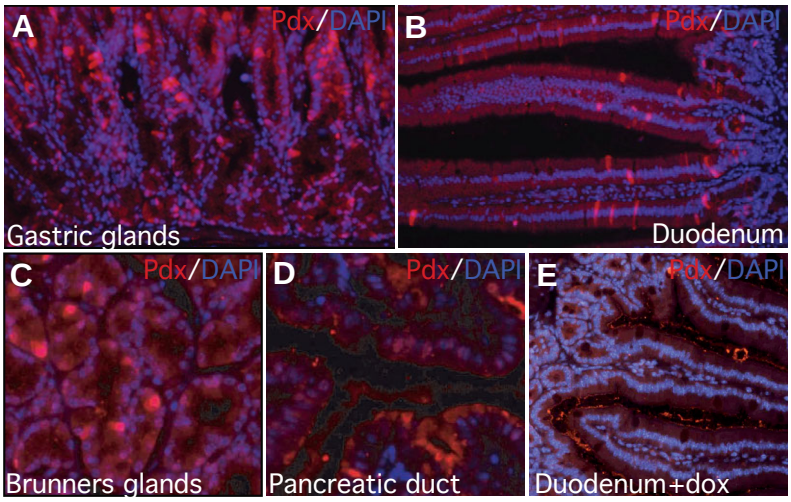

Fig. 2. Histological expression of Pdx1 in the adult gastro-duodenal domain. (A-E) *Immunofluorescence staining of Pdx1 (red) in gastric glands* **(A)**, *duodenal villi and crypts* **(B)**, *Brunner's glands* **(C),** *the pancreatic duct at the Ampulla of Vater* **(D).** *Pdx1 protein could not be detected in duodenal villi after treatment of mice for 14 days with doxycycline* **(E)**. *Nuclei are labelled with DAPI (blue).*

doxycycline, Pdx1 expression in the entire gastro-duodenal domain was undetectable by immunostaining (Fig. 2E).

Pdx1 is required to maintain positional identity in the gastro-duodenal domain

Previously, we tested the reversibility of doxycycline-mediated *Pdx1* repression. Following doxycyline withdrawal after 14 days treatment, elevated blood glucose concentrations decreased over 5-6 weeks concomitant with re-expression of *Pdx1* in pancreatic β cells (Holland *et al.*, 2005).

Examination of mice after *Pdx1* de-repression revealed abnormal gastro-duodenal morphology ranging from disorganisation of the rostral duodenal epithelia and Brunner's gland domain to the formation of hamartomatous polyps localised to the domain of the gastro-intestinal tract where *Pdx1* is normally expressed, namely the distal stomach (Fig. 3 A,A'), gastro-duodenal junction (Fig. 3B, C, C') and the peri-ampullar region of the duodenum (Fig. 3 B,B'). Lesions were observed in 7 of 8 mice examined following *Pdx1* repression and were never observed in untreated (n=19) or aged mice (n= 10; 560-650 days old).

The hamartomas exhibited features consistent with a process of intercalary regeneration, similar to the lesions observed in Cdx2 heterozygous mice (Chawengsaksophak *et al.*, 1997; Tamai *et al.*, 1999). In the example shown in Fig. 4, a pyloric hamartoma is sharply delineated by ectopic stratified squamous epithelia (SSE) at its anterior and posterior border. Between these borders, tissues resembling small intestine (Fig. 4B), pyloric glands (Fig. 4C) and glandular gastric tissue (Fig. 4D) are clearly evident. To further characterise the ectopic tissue we stained for the expression of Cdx2 which is normally expressed in intestinal epithelia (Fig. 5A) and for Sox2 (Fig. 5B) which shares a similar expression profile to Pdx1 in the Brunner's glands, pylorus and distal stomach, (Figs. 5B, C). The ectopic intestinal-type tissue expressed Cdx2 (Fig. 5D) and the pyloric-type tissue expressed Sox2 (Fig. 5E). Pdx1 (Fig.5F) was expressed between these domains of Cdx2 and Sox2 expression. In de-repressed mice Pdx1 expression was restored to relatively high levels in the pancreas but only low levels in the intestinal epithelia and Brunner's glands. We confirmed this low level of expression by co-localisation of the GFP protein encoded by the tetO-Pdx-IRES-GFP transgene (Holland *et al.*, 2005)(Fig. 5A-F). More posterior tissue types (e.g. colonic) were not observed in these lesions suggesting that the transformation is constrained by local factors to include only tissues normally found between the most anteriorised (stratified squamous epithelia (SSE)) and native (duodenal/small intestinal) domains. To determine if the hamartomas were proliferative, animals were treated with BrdU twice a week after withdrawal of doxycycline. The hamartomas were extensively labelled with BrdU confirming that they continued to proliferate during the recovery period. Cells in the outer SSE layer of a hamartoma did not incorporate BrdU, suggesting that this ectopic SSE arose prior to the withdrawal of doxycycline (Supplementary Fig. S1).

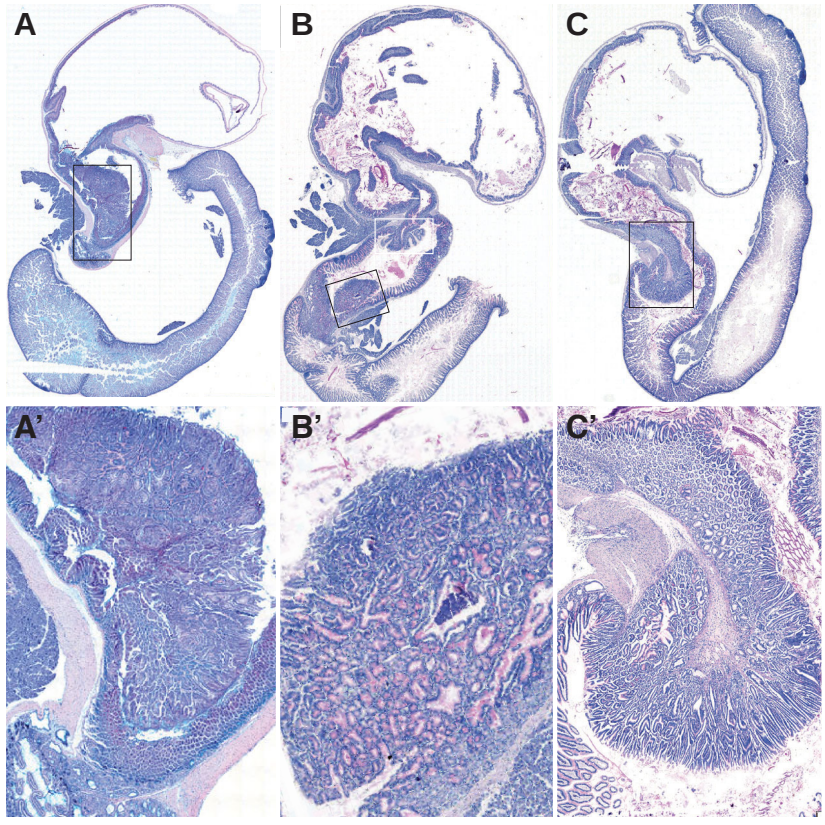

Fig. 3. Hamartoma formation in the gut of Pdx1-repressed mice. (A-C) *PAS stained sagittal sections of 3 mice treated with doxycycline for 14 days and examined 6 weeks later, showing hamartomas (boxed) in the distal stomach, pylorus and duodenum.* **(A'-C')** *High power images of the hamartomas shown in the areas delineated by the black boxes in (A-C).*

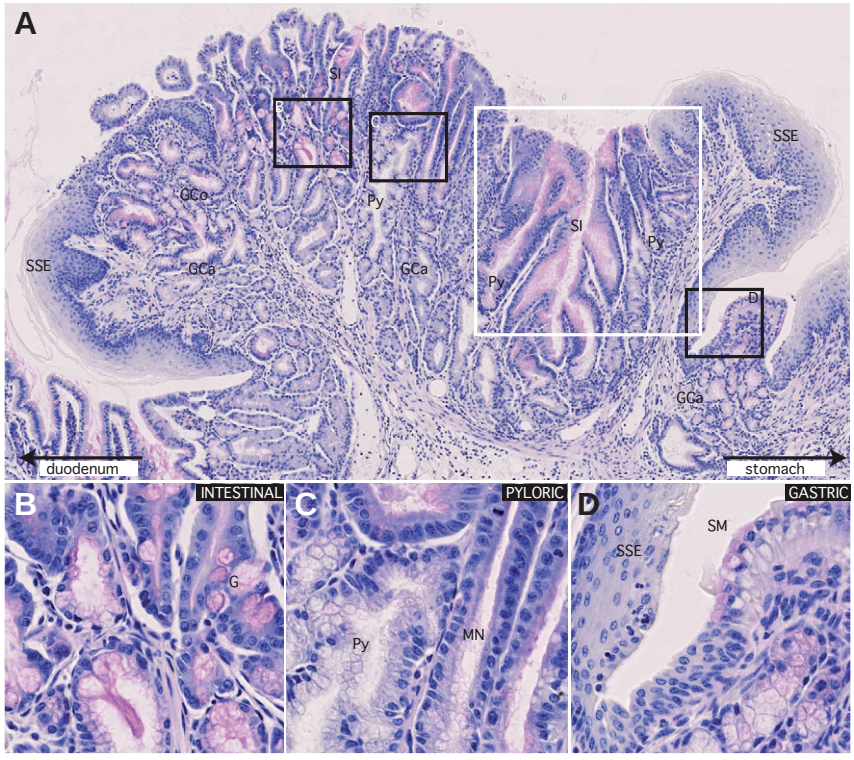

Fig. 4. Intercalary regeneration in hamartomas. *Detail of the hamartoma at the pylorus shown boxed in white in Fig. 3B.* **(A)** *Collage of a PAS-stained section of a pyloric hamartoma demonstrating transition from the stratified squamous epithelia, (SSE) through tissues resembling gastric cardia (GCa), gastric corpus (GCo) and pyloric (Py) and intestinal domains (SI).* **(B-D)** *Detail of the boxed areas in* **(A)** *reveals goblet cells (G), deep gastric pits and mucous neck cells (MN) indicative of pyloric glands (Py) and SSE transitioning to the surface mucous (SM) cells of the gastric cardia.*

Pdx1 repression alters Brunner's gland morphology and anteriorization of the rostral duodenum

To further investigate the early development of the hamartomatous lesions we examined untreated mice, mice treated with dox for 14d and mice treated with dox for 14d and then without dox for a further 14d (Fig. 6). Immunofluorescence staining of the stomach and small intestine confirmed absence of Pdx1 within 14 days of doxycycline administration (Fig. 2E and data not shown). No polyp-like lesions were observed in these mice, although histological examination revealed abnormal cellular organization in the gastro-duodenal domain. In untreated mice, the Brunner's gland acini were typically plump with a small luminal diameter (Fig.6A). Within 14 days of doxycycline administration, the glands had become cystic and were not sharply delineated from surrounding duodenal epithelia (Fig. 6B). Histological abnormalities were observed in 10 of 14 Pdx1 repressed mice examined and in 3 of these we could identify small foci of ectopic SSE in the distal stomach/pylorus as early as 14 days after doxycycline administration (Fig. 6C-E). The ectopic SSE expressed similar levels of Sox2 to that normally observed in the gastric fundus or esophageal SSE (Figure 6E-F). In addition to the abnormal organisation, the epithelia of the cystic Brunner's gland structures appeared to lose their typical pyramidal shape and basal polarity, assuming a more cuboidal or columnar phenotype (Fig. 6 B,G-I). After 14 days of recovery (14 days +dox, 14d no dox), we observed evidence of transformation adjacent to the cystic Brunner's glands domain, with esophageal, gastric and intestinal tissue types all present (Fig. 6 G-I).

Previous studies have shown that the number of enteroendocrine cells and enteroendocrine gene expression are reduced in the foregut of neonatal *Pdx1*-null mice (Chen *et al.*, 2009; Larsson *et al.*, 1996; Offield *et al.*, 1996). Histological examination of the proximal duodenum of adult mice in which *Pdx1* had been conditionally repressed revealed expression of serotonin, secretin and gastrin in the absence of Pdx1 (Supplementary Fig. S2 A-C). This indicates that Pdx1 is not essential for

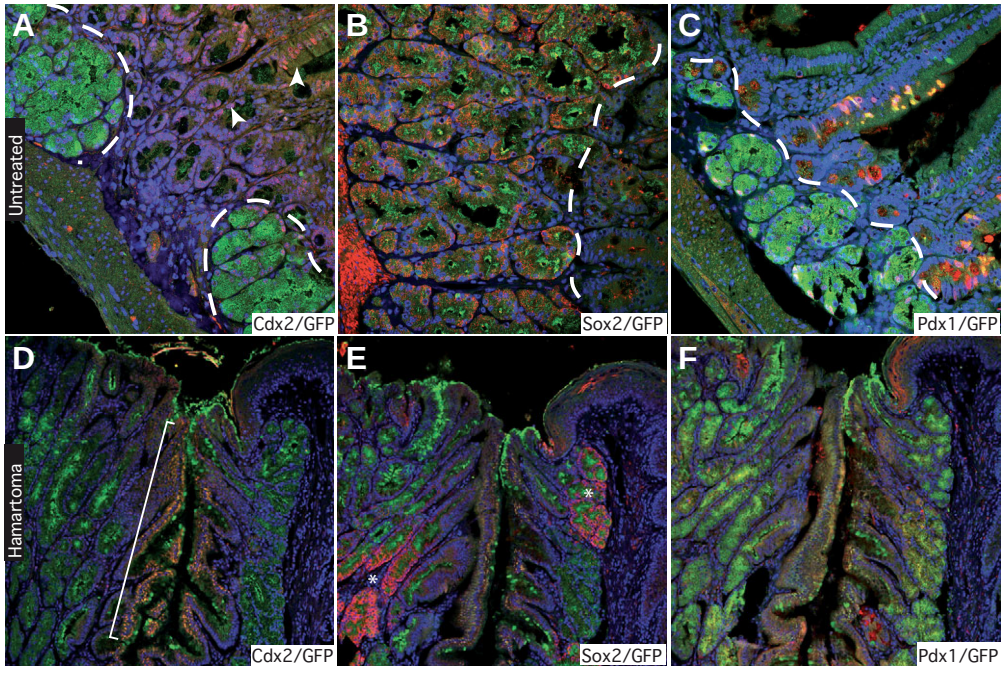

Fig. 5. Expression of tissue specific genes in a pyloric hamartoma. *Expression of Cdx2* **(A,D)**, *Sox2* **(B,E)** *and Pdx1* **(C,F)** *(red) in the Brunner's gland domain of an untreated mouse* **(A-C)** *and the white boxed area of the hamartoma shown in Figure 4* **(D-F)**. *Sox2 (*) and Cdx2 (bracketed) staining corresponds to areas identified histologically as pyloric/gastric and intestinal, respectively. Low-level Pdx1 expression was confirmed by immunostaining for GFP (green/yellow). Nuclei are labelled with DAPI (blue).*

the expression of these enterohormones or the maintenance of the enteroendocrine cells in the adult foregut. However, expression of gastrin in Brunner's glands appeared to be altered by the repression of Pdx1. In untreated mice, gastrin was detected homogeneously throughout the acini of the Brunner's glands (Supplementary Fig. S2D), whereas 14 days after doxycycline administration gastrin expression was weak, granular and localised to the perinuclear region (Supplementary Fig. S2E).

Discussion

The homeodomain transcription factor Pdx1 is well established as a critical regulator of foregut patterning during embryogenesis (reviewed in (Jørgensen *et al.*, 2007). In the present study we demonstrate that Pdx1 is also required in the adult mouse for maintenance of gastro-duodenal cell positioning and that the absence of Pdx1 leads to an anterior homeotic shift and hamartoma formation.

The Parahox gene cluster is a highly conserved paralogue of the classical Hox gene cluster comprising *Pdx1, Cdx2 and Gsh1*. Both the Hox and Parahox clusters are thought to have arisen from a common ancestral protohox cluster. The Parahox genes display temporal and spatial colinearity, a hallmark of the classical Hox genes, but unlike the predominantly ecto- or mesodermally expressed Hox genes, Parahox genes are expressed primarily in the endodermal germ layer and its derivatives. Although the evolutionary origin of the Hox and Parahox genes is still contentious, an intriguing model has been proposed in which the origin of the Parahox genes is linked to patterning of the endoderm and *Cdx2* and *Pdx1* interact to pattern the gut (Garcia-Fernàndez, 2005).

Developmentally, Pdx1 is required for the specification of the foregut. Loss of Pdx1 results in the arrest of pancreas development beyond the initial primordia, abnormal development of the pyloric sphincter and failure of the Brunner's glands to develop at the gastro-duodenal junction. Expansion of the Pdx1 domain through ectopic expression in the anterior or posterior endoderm represses Sox2 or Cdx2 expression, respectively, both markers of tissue identity in these domains (Grapin-Botton *et al.*, 2001; Offield *et al.*, 1996). This developmental patterning role for Pdx1 appears to be evolutionarily conserved as far back as the divergence of the bilaterians (Cole *et al.*, 2009). In the Sea Urchin, the Parahox gene homologs SpLox (Pdx1) and SpCdx are required to partition the posterior endoderm and for the development of a functional gut. Further, inhibition of SpLox was found to inhibit the Sea Urchins digestive ability and eliminated the constriction between the mid- and hindgut, suggesting a conserved role for Pdx1 in the correct development of the pylorus.

Loss of one Cdx2 allele during development results in the formation of polyp-like hamartomas in the rostral colon within the domain of highest Cdx2 expression (Beck *et al.*, 1999; Chawengsaksophak *et al.*, 1997; Tamai *et al.*, 1999). Beck *et al.*, (Beck *et al.*, 1999) hypothesised that a local deficiency in Cdx2 during development leads to anteriorisation of cells in the developing colon. The ectopic cell focus is then constrained by signals from surrounding tissues and through a process of intercalation forms a metaplastic growth or hamartoma comprising a gradient of tissue types from the anteriorised cells to the normal colonic phenotype. The phenotype of the gastro-duodenal domain that we observed after repressing Pdx1 is strikingly similar to that observed in the colon of Cdx2 heterozygous mice, leading us to propose a model in which the Parahox genes have a conserved functional role to

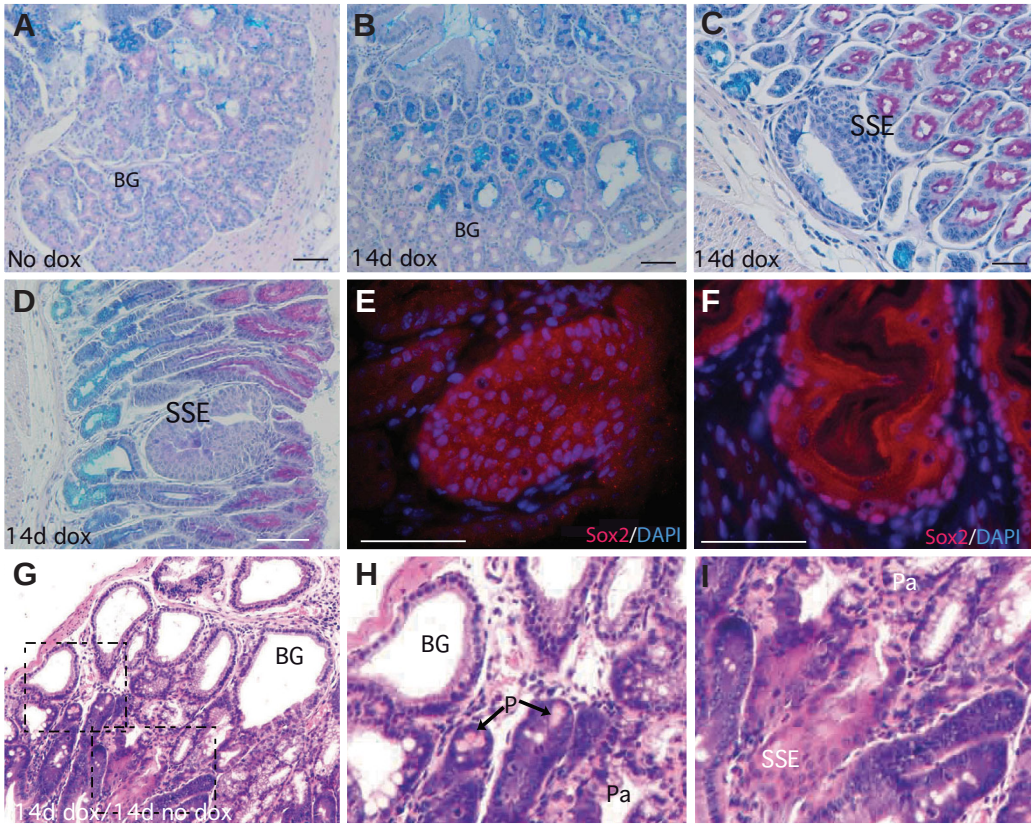

Fig. 6. Progressive loss of positional identity in the pylorus of Pdx1 repressed mice. (A) *PAS and Alcian blue staining of the pylorus in untreated mice demonstrate the plump, pale pink acini and clear delineation of Brunner's glands from the deep blue staining of the acidic intestinal mucins.* **(B)** *14 days after doxycycline administration the acini of Brunner's glands appear cystic and disorganised and* **(C-D)** *foci of SSE present in the distal stomach.* **(E)** *Serial section of the ectopic gastric SSE in* **(D)** *is positive for Sox2.* **(F)** *Typical Sox2 staining present in the SSE of the gastric fundus.* **(G)** *After a further 14d (-dox), the Brunners gland domain is highly cystic and disorganised.* **(H,I)**, *Higher resolution images of boxed areas in* **(G)** *shows characteristic eosinophilic granules of intestinal Paneth cells (P) apposed to a field of gastric parietal cells (Pa), as well as a focus of SSE and detail of the mixed columnar/cuboidal epithelia of the cystic Brunner's glands (BG). Bar in (D-F), 50 μm.*

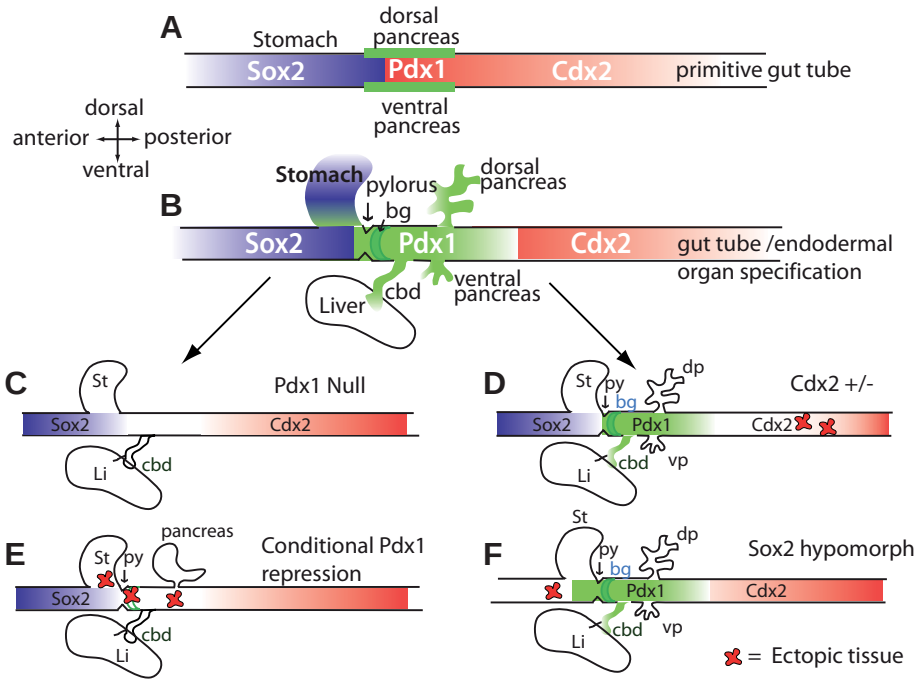

Fig. 7. A model for the role of Parahox genes in development and maintenance of the foregut. **(A)** *Following early patterning and specification of the gut tube, the homeodomain genes Cdx2 (red), Pdx1 (green) and Sox2 (blue) interact to partition the gut. Initially, the Sox2 and Cdx2 expression domains are closely apposed and interact to specify the anterior-posterior boundary.* **(B)***. Pdx1 is expressed dorsally and ventrally and acts to refine the anterior foregut domain before the Sox2 and Cdx2 expression domains recede anteriorly and posteriorly, respectively (Sherwood* et al.*, 2009). Loss of any of these patterning genes results in a loss of positional identity and the development of ectopic tissue within these domains (see text).* **(C-F)** *Absence of Pdx1 during development results in the almost complete loss of the pancreatic and pyloric domains whereas loss of Pdx1 in the adult, or Cdx2 or Sox2 haploinsufficency during development, causes the formation of anteriorized foci of metaplastic tissue consistent with a loss of positional identity. Li, liver; St, stomach; cbd, common bile duct; vp, ventral pancreas; dp, dorsal pancreas; py, pylorus; bg, Brunner's glands.*

maintain positional identity in the gut, both during development and in the adult (Fig. 7).

Unlike in Cdx2 heterozygotes, hamartomas have not been observed in heterozygous Pdx1 mice, implying greater tolerance for reduced Pdx1 concentrations in the anterior gut. This notion is supported by studies with hypomorphic Pdx1 mice that lack the conserved Area I-II-III regulatory region of the Pdx1 promoter, in which a reduction in the quality of Pdx1 expression led to impaired differentiation of enteroendocrine cells but no change in morphogenesis of the stomach, pylorus and rostral duodenum (Fujitani, 2006).

We previously demonstrated that repression of Pdx1 in adult mice impairs pancreatic beta-cell function leading to insulin deficiency and hyperglycemia. In the present study, we cannot eliminate the possibility that the observed phenotype is related to physiological disturbance secondary to the global repression of Pdx1. However, several lines of evidence lead us to conclude that hamartoma formation is a direct result of the induced loss of Pdx1 expression in the gastro-duodenal domain and, more specifically, in cells in which Pdx1 expression is relatively high. First, time-course observations from the onset of Pdx1 repression revealed the presence of transformed cells manifest as small foci of SSE followed by increasing disorganisation of the Brunner's gland epithelia. The identification of foci of SSE in the pylorus is consistent with the postulated mechanism of hamartoma formation in Cdx2 heterozygotes, in which colonic hamartomas took up to 6 weeks to develop and originated from localised SSE heterotopia (Beck *et al.*, 1999). Notably, this model is consistent with the notion of parahox genes conferring a posteriorizing code to a 'default' anterior patterned endoderm (Fig. 7). Second, a recent study in which Pdx1 was conditionally inactivated in villin-expressing intestinal cells demonstrated a requirement for Pdx1 to maintain gene expression in enterocytes and enteroendocrine cells, but metaplastic transformation of the gut was not observed (Chen *et al.*, 2009). As villin is not expressed in the Brunner's glands or gastric epithelia this study lends further weight to the hypothesis that Pdx1 is required to maintain positional identity in these cells and that the cellular transformation is initiated from these cells. Third, to our knowledge, intestinal hamartomas have not been reported in any of the other mouse models of diabetes in which mice are maintained in a chronically hyperglycaemic state (Atkinson and Leiter, 1999; Sutherland *et al.*, 2002). Finally, we did not observe hamartomas in any of the untreated but doxycycline-responsive mice or heterozygous littermates, making it unlikely that the genetic background of the mice was responsible.

Collectively, these observations suggest a model in which the Parahox genes play a classical Hox-like role in patterning the developing foregut endoderm and in maintaining foregut boundaries in adults (Fig. 7). In this model, a 'default' gut identity is initially specified early in development. Subsequently, the gut domains are delineated in response to the antagonistic anterior-posterior expression of the Parahox genes Cdx2 and Pdx1. If expression of these key genes falls below a threshold, a cell or cells may fail to acquire the correct positional identity resulting in the development of a foci of ectopic anteriorised tissue, representing in effect an anterior homeotic shift of the affected cells. In this regard, Cdx2 has recently been shown to actively repress the foregut differentiation program in the posterior gut and promote intestinal differentiation in the stomach, whereas Sox2 appears to be required for patterning of the anterior foregut as Sox2-deficient mice exhibit transformation of the anterior stomach and esophagus to intestinal and posterior stomach fates (Gao *et al.*, 2009; Long and Hornick, 2009; Que *et al.*, 2007; Sherwood *et al.*, 2009; Silberg *et al.*, 2002). Humans exhibit examples in which ectopic tissues arise either during embryogenesis (heterotopias) or post-natally (metaplasias). As most metaplasia is found in developmentally-adjacent tissues it has been proposed that the lesions represent the vertebrate manifestation of homeotic mutations in insects, whereby the expression of one or two 'master' transcription factors is sufficient to regulate the

fate of neighbouring tissues (Slack, 1985). Barrett's metaplasia (or Barrett's esophagus) is characterised by the transformation of the SSE lining the esophagus to intestinal-type epithelium. It is associated with trauma from chronic gastro-duodenal reflux and is considered a precursor to esophageal and gastro-esophageal carcinoma (Jankowski *et al.*, 2000). Cdx2 expression is associated with Barrett's metaplasia but does not appear sufficient to initiate the tissue transformation in the esophagus (Colleypriest *et al.*, 2010). Definitive identification of the cells responsible for the initiation of the hamartomas in either the present study or the Cdx2 haploinsuffiency model (Beck *et al.*, 1999) remain unclear. Identification of these cells and further delineation of the relationship of Pdx1, Cdx2 and Sox2 may lead to insights into the pathogenesis of metaplastic disorders such as Barrett's oesophagus.

In summary, our observations are consistent with and lend further weight to the models proposed by Tosh and Slack (Colleypriest *et al.*, 2010; Slack, 1985; Tosh and Slack, 2002), who postulated a generalised mechanism for transdifferentiation based on evidence in nature and experimental models. Our observations imply that the model can be extended (Fig. 7) to the maintenance of positional boundaries in the adult gut where Pdx1 continues to be expressed at high levels and suggest a conserved homeotic role for the Parahox genes in patterning of the endoderm and its derivatives in the adult as well as the developing gut.

Materials and Methods

Generation of knock-in and transgenic mice

Pdx1^{tTA} knock-in and Tg^{Pdx1} transgenic mice were generated as previously described (Holland *et al.*, 2002). To obtain mice in which *Pdx1* could be conditionally repressed, the *Pdx1*$^{tTA/+}$ knock-in mice were crossed with Tg^{Pdx1} mice and progeny intercrossed to derive *Pdx1*$^{tTA/tTA}$;Tg^{Pdx1} mice as well as genotype control mice (Supplementary Fig. S3).

Mouse care and treatment

Mice were housed under standard 12hr light/dark conditions and fed and watered *ad libitum*. Experiments were approved by The Royal Melbourne Hospital Campus Animal Research Ethics Committee. Doxycycline was administered as a single intraperitoneal dose of 100 mg/kg and maintained by addition to drinking water at 0.5 mg/ml. BrdU labelling was performed by twice weekly i.p injection of BrdU at 100mg/kg.

Immunocytochemistry and immunofluorescence

Tissues were fixed in Histochoice (Sigma, St. Louis, MO) or 4% paraformaldehyde for 3 hr to overnight before being processed, embedded in paraffin and sectioned (5 μm). Histological stains (hematoxylin and eosin, Periodic Acid Schiff and Alcian Blue) were performed by standard staining protocols. The following primary antibodies were used for immunofluorescence: rabbit anti-Pdx1 (a gift from Dr. Christopher Wright, Vanderbilt University Medical Center, Nashville, TN) at 1:500, rabbit anti-Cdx2 (Cell Signalling, Danvers, MA, USA), rabbit anti-Sox2 (Cell Signalling), chicken-anti GFP (Abcam, Cambridge MA, USA). Antibodies were detected with Alexafluor 568–conjugated goat anti-rabbit immunoglobulin and Alexafluor 488–conjugated goat anti-chicken immunoglobulin (Molecular Probes, Eugene, OR). Nuclei were counterstained with 4', 6'-diamidine-2'-phenylindole dihydrochloride (DAPI). BrdU incorporation was detected with mouse monoclonal anti-BrdU primary (Amersham, Buckinghamshire, UK) and Alexafluor 568 – conjugated anti- mouse secondary antibodies. Digital images were captured with an Axiocam camera from an Axioplan2 compound microscope (Zeiss) or Nikon C1 confocal microscope. Dual-color immunofluorescence images were compiled with NIH Image J software from two separate images of the same section using fluorochrome-specific filters.

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References

ATKINSON, M.A. and LEITER, E.H. (1999). The NOD mouse model of type 1 diabetes: as good as it gets? *Nat Med* 5: 601-604.

BECK, F., CHAWENGSAKSOPHAK, K., WARING, P., PLAYFORD, R. and FURNESS, J. (1999). Reprogramming of intestinal differentiation and intercalary regeneration in Cdx2 mutant mice. *Proc Natl Acad Sci USA* 96: 7318-7323.

BROOKE, N.M., GARCIA-FERNÀNDEZ, J. and HOLLAND, P.W. (1998). The ParaHox gene cluster is an evolutionary sister of the Hox gene cluster. *Nature* 392: 920-922.

CHAWENGSAKSOPHAK, K., JAMES, R., HAMMOND, V.E., KÖNTGEN, F. and BECK, F. (1997). Homeosis and intestinal tumours in Cdx2 mutant mice. *Nature* 386: 84-87.

CHEN, C., FANG, R., DAVIS, C., MARAVELIAS, C. and SIBLEY, E. (2009). Pdx1 Inactivation Restricted to the Intestinal Epithelium in Mice Alters Duodenal Gene Expression in Enterocytes and Enteroendocrine Cells. *Am J Physiol Gastrointest Liver Physiol* 297: G1126-G1137.

COLE, A.G., RIZZO, F., MARTINEZ, P., FERNANDEZ-SERRA, M. and ARNONE, M.I. (2009). Two ParaHox genes, SpLox and SpCdx, interact to partition the posterior endoderm in the formation of a functional gut. *Development* 136: 541-549.

COLLEYPRIEST, B.J., FARRANT, J.M., SLACK, J.M.W. and TOSH, D. (2010). The role of Cdx2 in Barrett's metaplasia. *Biochem Soc Trans* 38: 364-369.

FERRIER, D.E.K., DEWAR, K., COOK, A., CHANG, J.L., HILL-FORCE, A. and AMEMIYA, C. (2005). The chordate ParaHox cluster. *Curr Biol* 15: R820-R822.

FUJITANI, Y. (2006). Targeted deletion of a cis-regulatory region reveals differential gene dosage requirements for Pdx1 in foregut organ differentiation and pancreas formation. *Genes Dev.* 20: 253-266.

FUKUDA, A., KAWAGUCHI, Y., FURUYAMA, K., KODAMA, S., KUHARA, T., HORIGUCHI, M., KOIZUMI, M., FUJIMOTO, K., DOI, R. and WRIGHT, C. (2006). Loss of the Major Duodenal Papilla Results in Brown Pigment Biliary Stone Formation in Pdx1 Null Mice. *Gastroenterology* 130: 855-867.

GAO, N., WHITE, P. and KAESTNER, K.H. (2009). Establishment of intestinal identity and epithelial-mesenchymal signaling by Cdx2. *Dev. Cell* 16: 588-599.

GARCIA-FERNÀNDEZ, J. (2005). The genesis and evolution of homeobox gene clusters. *Nat Rev Genet* 6: 881-892.

GAUNT, S.J., DRAGE, D. and TRUBSHAW, R.C. (2008). Increased Cdx protein dose effects upon axial patterning in transgenic lines of mice. *Development* 135: 2511-2520.

GRAINGER, S., SAVORY, J.G. and LOHNES, D. (2010). Cdx2 regulates patterning of the intestinal epithelium. *Dev Biol* 339: 155-165.

GRAPIN-BOTTON, A., MAJITHIA, A. and MELTON, D. (2001). Key events of pancreas formation are triggered in gut endoderm by ectopic expression of pancreatic regulatory genes. *Genes Dev* 15: 444-454.

HALE, M., KAGAMI, H., SHI, L., HOLLAND, A.M., ELSASSER, H., HAMMER, R. and MACDONALD, R.J. (2005). The homeodomain protein PDX1 is required at mid-pancreatic development for the formation of the exocrine pancreas. *Dev Biol* 286: 225-237.

HOLLAND, A.M., GÓÑEZ, L.J., NASELLI, G., MACDONALD, R.J. and HARRISON, L.C. (2005). Conditional expression demonstrates the role of the homeodomain transcription factor Pdx1 in maintenance and regeneration of beta-cells in the adult pancreas. *Diabetes* 54: 2586-2595.

HOLLAND, A.M., HALE, M., KAGAMI, H., HAMMER, R. and MACDONALD, R.J. (2002). Experimental control of pancreatic development and maintenance. *Proc Natl Acad Sci U SA* 99: 12236-12241.

JANKOWSKI, J.A., HARRISON, R.F., PERRY, I., BALKWILL, F. and TSELEPIS, C. (2000). Barrett's metaplasia. *Lancet* 356: 2079-2085.

JØRGENSEN, M.C., AHNFELT-RØNNE, J., HALD, J., MADSEN, O.D., SERUP, P. and HECKSHER-SØRENSEN, J. (2007). An illustrated review of early pancreas development in the mouse. *Endocr Rev* 28: 685-705.

KAWAGUCHI, Y., COOPER, B., GANNON, M., RAY, M., MACDONALD, R.J. and WRIGHT, C. (2002). The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. *Nat Genet* 32: 128-134.

LARSSON, L.I., MADSEN, O.D., SERUP, P., JONSSON, J. and EDLUND, H. (1996). Pancreatic-duodenal homeobox 1 -role in gastric endocrine patterning. *Mech. Dev.* 60: 175-184.

LONG, K.B. and HORNICK, J.L. (2009). SOX2 is highly expressed in squamous cell carcinomas of the gastrointestinal tract. *Human Pathology* 40: 1768-1773.

OFFIELD, M.F., JETTON, T.L., LABOSKY, P.A., RAY, M., STEIN, R.W., MAGNUSON, M.A., HOGAN, B.L. and WRIGHT, C.V. (1996). PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development* 122: 983-995.

QUE, J., OKUBO, T., GOLDENRING, J.R., NAM, K.-T., KUROTANI, R., MORRISEY, E.E., TARANOVA, O., PEVNY, L.H. and HOGAN, B.L.M. (2007). Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm. *Development* 134: 2521-2531.

SHERWOOD, R.I., CHEN, T.-Y.A. and MELTON, D.A. (2009). Transcriptional dynamics of endodermal organ formation. *Dev Dyn* 238: 29-42.

SILBERG, D., SULLIVAN, J., KANG, E., SWAIN, G., MOFFETT, J., SUND, N., SACKETT, S. and KAESTNER, K. (2002). Cdx2 ectopic expression induces gastric intestinal metaplasia in transgenic mice. *Gastroenterology* 122: 689-696.

SLACK, J.M. (1985). Homoeotic transformations in man: implications for the mechanism of embryonic development and for the organization of epithelia. *J Theor Biol* 114: 463-490.

STANGER, B.Z., DATAR, R., MURTAUGH, L.C. and MELTON, D.A. (2005). Direct regulation of intestinal fate by Notch. *Proc Natl Acad Sci USA* 102: 12443-12448.

SUTHERLAND, R.M., MOUNTFORD, J.N., ALLISON, J., HARRISON, L.C. and LEW, A.M. (2002). The non-immune RIP-Kb mouse is a useful host for islet transplantation, as the diabetes is spontaneous, mild and predictable. *Int J Exp Diabetes Res* 3: 37-45.

TAMAI, Y., NAKAJIMA, R., ISHIKAWA, T., TAKAKU, K., SELDIN, M.F. and TAKETO, M.M. (1999). Colonic hamartoma development by anomalous duplication in Cdx2 knockout mice. *Cancer Research* 59: 2965-2970.

TOSH, D. and SLACK, J.M.W. (2002). How cells change their phenotype. *Nat Rev Mol Cell Biol* 3: 187-194.

TWYMAN, R.M. (2001). *Dev. Biol.* BIOS Scientific, Leeds.

ZORN, A.M. and WELLS, J.M. (2009). Vertebrate endoderm development and organ formation. *Annu. Rev. Cell. Dev. Biol.* 25: 221-251.

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