

REVIEW

Stomach development, stem cells and disease

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ABSTRACT

The stomach, an organ derived from foregut endoderm, secretes acid and enzymes and plays a key role in digestion. During development, mesenchymal-epithelial interactions drive stomach specification, patterning, differentiation and growth through selected signaling pathways and transcription factors. After birth, the gastric epithelium is maintained by the activity of stem cells. Developmental signals are aberrantly activated and stem cell functions are disrupted in gastric cancer and other disorders. Therefore, a better understanding of stomach development and stem cells can inform approaches to treating these conditions. This Review highlights the molecular mechanisms of stomach development and discusses recent findings regarding stomach stem cells and organoid cultures, and their roles in investigating disease mechanisms.

KEY WORDS: Epithelial-mesenchymal interactions, Organogenesis, Transcriptional control of development

Introduction

A stomach is a muscular and characteristically curved portion of the proximal alimentary canal that is present in all jawed vertebrates that require food storage or preliminary digestion in an acidic environment. Originating from the foregut endoderm, the stomach epithelium becomes regionalized along the proximal-distal axis during development, giving rise to distinct functional regions or chambers. The forestomach in rodents, for example, develops a stratified squamous epithelium contiguous with the esophageal mucosa and it functions in the storage and mechanical digestion of food. By contrast, the glandular stomach has a simple columnar epithelium and is further divided into the corpus, which secretes acid and digestive enzymes and the antrum, which secretes mucus and certain hormones, particularly gastrin (San Roman and Shivdasani, 2011). To accommodate dietary variations, stomach size and shape vary widely among vertebrate species, and the various functional compartments occupy different fractions of the organ (Fig. 1). For example, the forestomach is absent in humans, but occupies the characteristic upper curvature or fundus region of the mouse stomach; the first three chambers in ruminant mammals have a similar stratified epithelium. In the avian stomach, an additional proximal glandular compartment known as the proventriculus (PV) secretes digestive enzymes while a distal gizzard (GZ) serves a mechanical grinding function (Romanoff, 1960).

A principal function of the stomach is to create an acidic milieu. Luminal acid secretion is estimated to have first occurred about 350 million years ago (Barrington, 1942), expanding both dietary

sources and barriers to pathogen entry because a low pH helps absorb metals from plant sources, denatures proteins, and kills microbes (Koelz, 1992). Luminal acidity is generated by H⁺/K⁺-ATPase proton pumps, which are expressed in dedicated oxyntic cells in the mammalian stomach and in bifunctional oxynto-peptic cells in lower vertebrates. Other gastric functions are to secrete mucins, acid-activated pro-peptidases (pepsinogens) and hormones that regulate responses to food or starvation. Genome analyses correlate loss of H⁺/K⁺-ATPase and pepsinogens with loss of a stomach in some vertebrate species during evolution, highlighting the significance of acid-peptic digestion (Castro et al., 2014). In contrast to this physiological function, stomach acidity contributes to considerable human morbidity and, coupled with environmental factors such as *Helicobacter pylori*, promotes peptic ulcers, esophageal reflux and gastric cancer – the third most common cause of worldwide cancer mortality. The dysregulation of developmental programs that produce an adaptive and functioning stomach may also underlie conditions such as intestinal metaplasia, a common bedfellow of chronic gastritis (Correa, 1988). Obtaining a detailed understanding of the signaling pathways that control stomach development will thus aid approaches to treat these diseases. In addition, a better understanding of the mechanisms that regulate gastric homeostasis and of the stem cells that underlie this regulation will facilitate the identification of better biomarkers and therapies.

Here, we review the molecular mechanisms of stomach specification, patterning and differentiation. We also discuss recent findings relating to gastric stem cell identity and function, highlighting how alterations in stomach development and stem cells might contribute to some human disorders.

Formation and regionalization of the definitive endoderm

Epiblast cells, which migrate through the primitive streak during gastrulation, were once believed to form definitive endoderm by displacing the visceral endoderm (Lawson et al., 1986; Tam and Beddington, 1987). However, live imaging coupled with genetic labeling demonstrates that some progeny of visceral endodermal cells mix with definitive endodermal cells, revealing both embryonic and extra-embryonic origins of the gut endoderm (Kwon et al., 2008). By the end of gastrulation, this undifferentiated endoderm is pre-patterned into three regions along the anterior-posterior axis: the foregut, which gives rise to the esophagus, trachea, lungs, liver, pancreas, hepatobiliary system and stomach; and the midgut and hindgut, which develop into the small and large intestines, respectively. This pre-patterning is evident from the restricted expression domains of transcription factors (TFs) and signaling receptors that later establish regionalization. Subsequently, the definitive endoderm develops into the epithelial lining of the stomach and other digestive organs. Abutting this epithelium is a connective tissue called the lamina propria; smooth muscle develops beneath the lamina propria and a thin layer of serosa forms the outermost radial layer. These sub-epithelial layers collectively originate in the splanchnic mesoderm,

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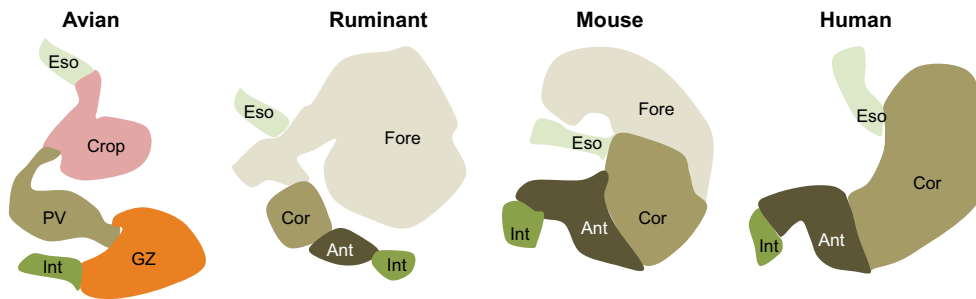


Fig. 1. Stomach anatomy. Illustration of the different stomach regions (or chambers) in birds and mammals. Eso, esophagus; GZ, gizzard; Fore, forestomach; PV, proventriculus; Int, intestine.

which associates early with the undifferentiated gut tube, whereas the enteric nervous system derives from neural crest cells that subsequently migrate into the sub-epithelium. The tightly coordinated development of these endoderm and mesoderm derivatives is necessary for proper stomach organogenesis.

In embryos, various TFs and intercellular signals provide the cell-intrinsic and non-cell autonomous means, respectively, for the stomach to form precisely between the esophagus and small intestine (Fig. 2). For example, TFs such as HHEX and SOX2 are required in various capacities for proper foregut development (Dufort et al., 1998; Martinez Barbera et al., 2000; Que et al., 2009) and retinoic acid (RA) signaling is necessary for foregut organogenesis and to maintain the foregut-midgut boundary; accordingly, mouse embryos lacking *Raldh2* (also known as *Aldh1a2*), which encodes an enzyme involved in RA synthesis, show stomach defects in addition to lung, pancreas and liver anomalies (Molotkov et al., 2005; Wang et al., 2006). Signaling through the fibroblast growth factor (FGF) and Wnt pathways specifies hindgut endoderm and represses foregut fates (Fig. 2). Highlighting the evolutionary conservation of this patterning mechanism, signaling through FGF4 in mice induces posterior endoderm markers in a concentration-dependent manner (Wells and Melton, 2000) and gain- and loss-of-function studies in chick embryos demonstrate that FGF4 promotes the expression of midgut genes at the expense of foregut genes (Dessimoz et al., 2006). Similarly, canonical Wnt signaling is essential for hindgut development and its activity posteriorizes the foregut in mice and *Xenopus* (Gregorieff et al., 2004; McLin et al., 2007; Sherwood et al., 2011). In addition, gradients of bone morphogenetic proteins (BMPs) and secreted BMP antagonists pattern the endoderm along the anterior-posterior axis in many vertebrate species, whether the foregut gives rise to a distinct stomach or not (Tiso et al., 2002). In summary, specific signaling pathways combine to regionalize the

gut endoderm in diverse species, in part by restricting key TFs to particular domains; the understanding of the precise local actions of these pathways remains incomplete.

Stomach specification and regionalization

Following its specification, the early gut endoderm diverges into distinct organ primordia. Gene expression profiles and immunofluorescence analyses have mapped the dynamics of crucial organ-specific TFs in this process. Notably, the canonical TFs implicated in intestine development – CDX1 and CDX2 – are highly restricted to the intestinal endoderm in mid- and late gestation, whereas those implicated in stomach development (e.g. SOX2) tend also to be expressed in lung and esophageal endoderm (Sherwood et al., 2009). This suggests the presence of a common foregut progenitor cell pool and highlights that few if any regionally restricted TFs function exclusively in stomach development. Thus, whereas *Cdx2*, which is required for intestine specification (Gao et al., 2009; Grainger et al., 2010), is expressed selectively in the prospective mouse intestine, *Sox2* levels are high in embryonic esophageal and stomach epithelia, and reduced *Sox2* levels lead to defective differentiation of both tissues (Que et al., 2009). Conversely, ectopic *Sox2* expression in the mouse intestinal epithelium causes defective intestinal differentiation with activation of some gastric markers (Raghoebir et al., 2012), while forced *Cdx2* expression in the mouse stomach endoderm induces intestinal differentiation (Silberg et al., 2002). Moreover, *Cdx2*-null adult mouse intestinal stem cells thrive in culture conditions that promote gastric rather than intestinal differentiation (Simmini et al., 2014).

Although such findings suggest that the counterbalance of these two organ-specific TFs generates the sharp boundary between the posterior stomach and proximal intestine (Figs 2 and 3), the reality is probably more nuanced. Stomach specification per se is undisturbed

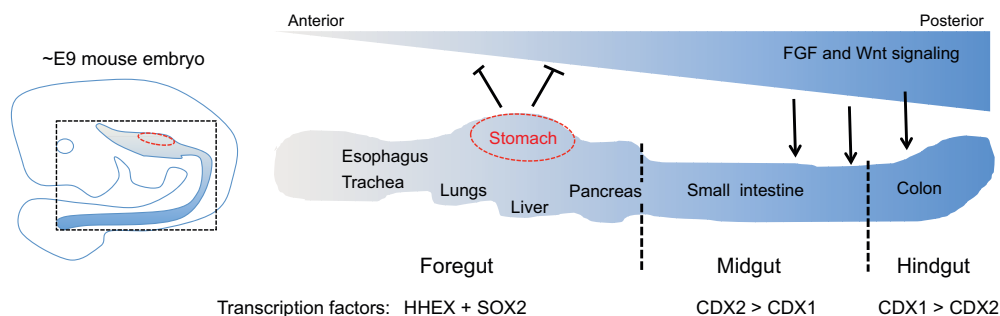


Fig. 2. Transcription factors and signaling pathways implicated in the regionalization of gut endoderm. Schematic illustration (left) of a mouse embryo at E9 highlighting the position of the prospective stomach (red circle). Early gut regionalization (right) is mediated by key TFs and intercellular signals: SOX2 and HHEX are essential for foregut development, whereas CDX1 and CDX2 are required in the midgut and hindgut; signaling through the FGF and Wnt pathways posteriorizes gut endoderm and the regional attenuation of these signals promotes stomach development.

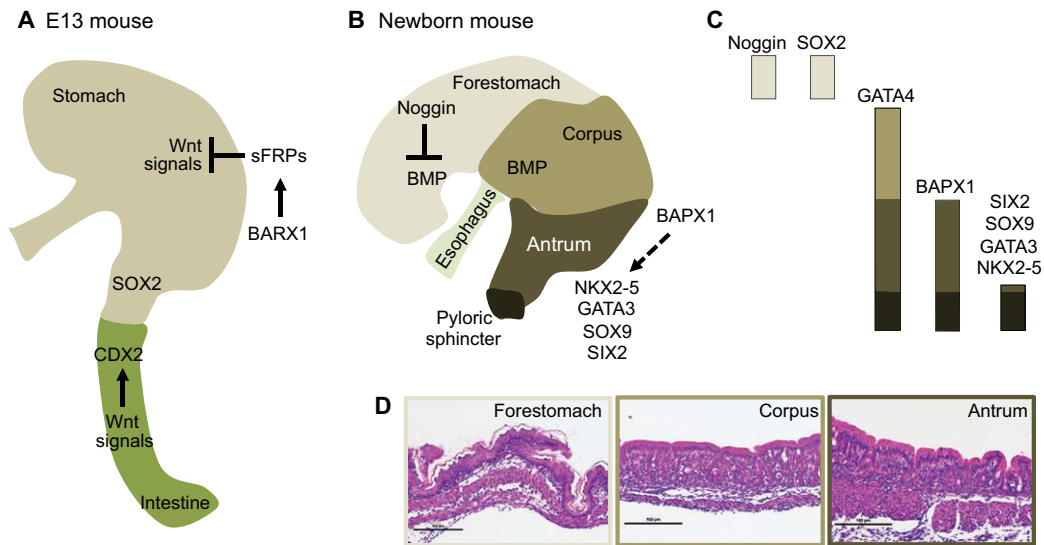


Fig. 3. Stomach patterning. Diagrams of the E13 (A) and newborn (B) mouse stomach. (A) Before regionalization, the entire stomach epithelium is pseudostratified. The transcription factors SOX2 and CDX2 define the sharp boundaries of the prospective stomach and intestine, possibly through mutual cross-antagonism. BARX1 is expressed specifically in mid-gestation stomach mesenchyme and induces secreted Wnt antagonists (sFRPs) to attenuate Wnt signaling, which ordinarily promotes intestinal development, in the overlying stomach epithelium. (B) Later, the mouse stomach differentiates into the forestomach, which has a stratified epithelium, and the glandular stomach, which has a columnar epithelium and contains two prominent regions: a rostral corpus and a caudal antrum. The most distal portion of the antrum forms a specialized muscular valve, the pyloric sphincter. (C) Signals and TFs implicated in newborn mouse stomach patterning. Noggin, which is highly expressed in the forestomach, restricts BMP signaling to the glandular stomach, where the TF genes *Gata4* and *Bapx1* are required for proper cellular development and morphogenesis. BAPX1 might regulate *Nkx2-5*, *Gata3*, *Sox9* and *Six2*, TF genes that are restricted to the distal antrum and necessary for development of the pyloric sphincter. (D) Hematoxylin and eosin stained histological sections of the newborn mouse stomach illustrate the stratified epithelium of the forestomach and the columnar epithelium of the corpus and antrum regions of the glandular stomach.

in mice with reduced *Sox2* expression (Que et al., 2009), although this might reflect persistent *Sox2* expression or redundancy with other factors, such as *Sox21*. More pertinently, *Cdx2* deletion in the early mouse endoderm results in colonic atresia and esophageal features in the distal intestine, but barely affects the gastro-intestinal junction or proximal intestine (Gao et al., 2009; Grainger et al., 2010). In addition, distinctive polyps with mixed gastric and intestinal features are confined to the distal midgut in *Cdx2*^{+/-} mice (Chawengsaksophak et al., 1997). Thus, although the absence of *Cdx2* might enable stomach differentiation, it is hardly sufficient; although CDX1 activity might compensate when CDX2 is missing, stomach development does not appear to be a simple sequela of *Cdx2* absence. Moreover, whereas prolonged loss of *Cdx2* from intestinal stem cells impairs intestinal differentiation (Stringer et al., 2012), *Cdx2* inactivation in adult mice does not significantly activate stomach-specific genes (Verzi et al., 2010).

The boundary between the stomach and pancreas is also created by particular TFs. Deletion of *Hes1* in the mouse causes ectopic pancreas development in the stomach through activation of the TF gene *Ptf1a* (Fukuda et al., 2006) and forced expression of *Ptf1a* converts stomach tissue to pancreas (Jarikji et al., 2007; Willet et al., 2014). Therefore, *Hes1*-mediated Notch signaling and its control over *Ptf1a* are required for proper specification of these organs. Conversely, absence of the POU-homeobox TF HNF1B results in expansion of the rostral and mid-stomach at the expense of the antrum and pancreas (Haumaitre et al., 2005). *Pdx1*, which encodes a TF best known for its functions in the pancreas, is also expressed in the gastric antrum and proximal duodenum, and has important dosage-dependent requirements in the specification and morphogenesis of these structures (Fujitani et al., 2006). In summary, TFs such as SOX2, CDX2, HNF1B, PDX1 and PTF1A play vital roles in the development of adjacent digestive organs and

in the cell-autonomous maintenance of epithelial fates, but our understanding of their mechanisms is incomplete and it remains unclear how their expression domains are restricted with exquisite precision.

Endodermal-mesenchymal interactions are also important in early stomach patterning and regionalization. Heterotopic xenografts of embryonic day (E)14 rat stomach endoderm and intestinal mesoderm develop with gastric features (Duluc et al., 1994) implying that, by this stage of development, positional information is programmed in the endoderm despite the absence of overt cytodifferentiation. However, grafting experiments prior to the equivalent developmental stage in chick embryos demonstrate crucial requirements for the underlying mesenchyme in stomach epithelial development (Koike and Yasugi, 1999). Arguably the best-studied factor for this instructive role is the homeodomain TF BARX1, which, among digestive organs, is expressed exclusively in the stomach and esophageal mesenchyme. The digestive tract in *Barx1*^{-/-} embryos is dramatically posteriorized, with intestinal villus cell types present in the stomach and a poor stomach-intestinal boundary (Kim et al., 2005, 2007). Forced *Barx1* expression in intestinal mesenchyme expands the smooth muscle compartment, producing muscle layers of a gastric type, but does not induce a stomach-type mucosa, indicating that additional, unknown factors are necessary to over-ride intestinal epithelial specification (Jayewickreme and Shivdasani, 2015). Cultured *Barx1*-deficient mesenchymal cells and *Barx1*^{-/-} embryos provide a useful clue into the identity of such factors: BARX1 is necessary for the expression of secreted Wnt antagonists, thereby inhibiting local Wnt signaling, and these Wnt antagonists also rescue the defects associated with *Barx1*-deficient stomach mesenchymal cells cultured *ex vivo* (Kim et al., 2005). Thus, the attenuation of Wnt signaling, which promotes intestinal development, is necessary in the proximal

alimentary canal for non-cell autonomous stomach specification (Fig. 3A).

After stomach specification, several other TFs are involved in stomach regionalization and patterning. The pseudo-stratified epithelium in the embryonic mouse stomach differentiates into two principal derivatives along the proximal-distal axis: the forestomach and the glandular stomach (Fig. 3B–D). The glandular stomach differentiates further into three areas: the cardia at the esophagus-stomach junction, the corpus for most stomach functions, and most distally, the antrum. Recent studies show that epithelial and mesenchymal TFs differentially expressed along the proximal-distal stomach axis pattern organ morphology as well as these regional epithelia (Fig. 3C). For example, in addition to dramatic defects in the gastric mucosa, *Barx1*^{-/-} embryos show marked fundic hypoplasia, resulting in abnormal stomach curvature (Kim et al., 2005). SOX2 is more abundant in forestomach than in glandular stomach epithelial cells, and reduced SOX2 levels prominently affect forestomach differentiation, with ectopic expression of genes specific to the glandular stomach (Que et al., 2009) (Fig. 3B,C). By contrast, the zinc-finger TF GATA4 is highly expressed in the developing glandular stomach, among other gut epithelia, and *Gata4*-null epithelial cells fail to contribute to this tissue in chimeric mice (Jacobsen et al., 2002), suggesting a role in stomach mucosal specification. The absence of another mouse homeodomain TF gene, *Bapx1*, which is expressed principally in the caudal (antral) stomach mesenchyme, causes truncation of the antrum and distorts distal stomach morphogenesis (Verzi et al., 2009). The homeobox TF HOXA5 is also strongly expressed in the hindstomach mesenchyme and required for its proper development (Aubin et al., 2002).

At the boundary with the proximal intestine, the antrum forms the pyloric sphincter, a muscular valve that is dilated in *Bapx1*^{-/-} mice. At least three other TFs – NKX2.5, GATA3 and SOX9 – are expressed in various combinations in undifferentiated cells in the pyloric mesenchyme, with *Sox9* expression partially dependent on the others (Self et al., 2009; Udager et al., 2014) (Fig. 3B). Loss of *Nkx2-5* or *Gata3* alters sphincter morphology as a result of severe hypoplasia of a particular dorsal fascicle of longitudinal smooth muscle (Udager et al., 2014). These findings collectively highlight the importance of regionally restricted TFs in stomach development, with loss of single factors often manifesting in both mesenchymal and epithelial defects. Additional TFs with potent patterning activity surely remain to be identified, as do mechanisms for TF cooperativity, antagonism and precise regional expression.

Epithelial-mesenchymal signaling during stomach development

Recombination cultures and viral misexpression studies in chick embryos have elegantly demonstrated the instructive effect of mesenchymal cells on overlying epithelia (Roberts et al., 1998; Fukuda and Yasugi, 2005). The co-culture of undifferentiated chick stomach endoderm with PV mesenchyme induces enzyme-secreting glands of the PV type, whereas culture with GZ mesenchyme inhibits the PV fate (Mizuno et al., 1986). Regionally restricted BMP ligands and antagonists are responsible for some of this effect and they particularly illustrate the recurrent use of the same signaling pathway to achieve distinct outcomes at different stages and locations in stomach development. In chick embryos, for example, BMP2 localizes to the PV mesenchyme and its overexpression increases the number of stomach glands, whereas ectopic expression of the BMP inhibitor noggin prohibits gland formation. This role for BMP signaling is, in part, conserved; the

mouse forestomach epithelium expresses BMP antagonists, effectively confining BMP signals to the glandular epithelium (Fig. 3B) and deletion of *Noggin* or ectopic BMP activation disrupts forestomach differentiation (Rodriguez et al., 2010). Another action of BMP signaling in the chick GZ mesenchyme is to activate *Sox9*, which, in turn, induces SOX9-dependent pyloric features in the overlying epithelium (Smith et al., 2000; Theodosiou and Tabin, 2005). In other tissue interactions, *Fgf10* and its receptor *Fgfr2* show reciprocal expression in the mouse mesenchyme and epithelium, respectively and the corresponding mutants display significant defects in growth of the glandular stomach, with especially reduced epithelial cell proliferation (Spencer-Dene et al., 2006); conversely, FGF10 hyperactivity expands the epithelium (Nyeng et al., 2007). Thus, stomach patterning and growth are mediated by tissue-specific ligand-receptor interactions in signaling pathways that are widely active, emphasizing the need to understand how these signals elicit distinct outcomes in diverse tissues. Although BMP-mediated activation of *Sox9* expression is well established, an outstanding question is how this and other pathways influence the expression and activities of other regionally restricted TFs in the developing stomach.

Stomach growth and morphogenesis are coupled and epithelial-mesenchymal crosstalk – in particular that mediated by the planar cell polarity (PCP) and hedgehog (Hh) signaling pathways – is involved in this coupling. The PCP pathway is particularly required for forestomach elongation. In mice lacking secreted frizzled-related protein (SFRP) family Wnt antagonists, the forestomach is truncated, with disturbed orientation of epithelial cell divisions, even though canonical Wnt signaling is intact; the same defect appears in mice lacking the core PCP component VANGL2 or its ligand WNT5A, which are expressed in the gut epithelium and mesenchyme, respectively (Matsuyama et al., 2009). Hh signaling controls growth of the whole alimentary tract through epithelium-mesenchyme interactions. *Shh* and *Ihh* expressed in the endoderm signal to the adjacent mesenchyme (Bitgood and McMahon, 1995; Kolterud et al., 2009). The deletion of both ligands causes significant attrition of mesenchymal cell populations, leading to severe growth defects and markedly diminished stomach size, although rostro-caudal patterning is remarkably preserved (Mao et al., 2010). Not all the mechanisms that underlie mesenchymal dependence on Hh signaling are known, but one effect is to modulate Notch signaling (Kim et al., 2011). Both activation and inhibition of the Notch pathway deplete the stomach mesenchyme, similar to the effect of Hh inhibition, and the addition of recombinant SHH to cultured fetal gut mesenchymal cells rescues Notch-induced cell death, revealing crosstalk between these signaling pathways in the developing stomach (Kim et al., 2011). Thus, in the highly coordinated process of stomach specification, patterning and growth, selected TFs respond to exchange of spatially and temporally controlled signals between the epithelium and mesenchyme.

Stomach differentiation

Epithelial (mucosal) differentiation

On the basis of histology, ultrastructure and specific products, five distinct differentiated cell types can be identified in the adult corpus, the dominant functional region (Fig. 4): foveolar (pit) cells, located at the top of stomach glands, produce mucus and turn over every 3 days; zymogenic (chief) cells at the bottom of the glands secrete digestive enzymes such as pepsinogen and turn over every few months; abundant parietal (oxyntic) cells along the gland shaft secrete HCl; endocrine cells, which account for <2% of the epithelium, secrete hormones; and finally tuft cells, which are just as

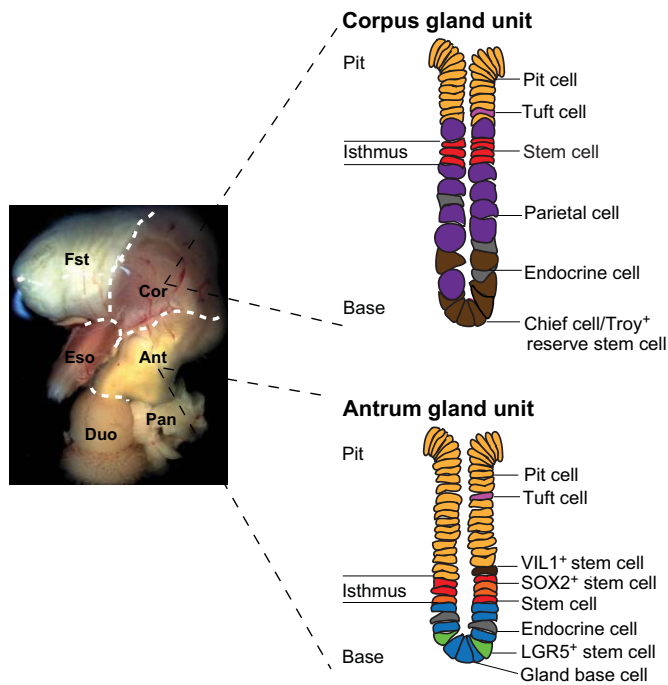


Fig. 4. Stomach mucosal lineages and stem cells. The adult mouse stomach is shown on the left (modified from Kim and Shivdasani, 2011). Corpus and antral gland units are depicted on the right. Each gland unit contains pit, isthmus and base regions. In the corpus, unidentified stem cells give rise to five principal cell types: mucus-producing pit cells, acid-secreting parietal cells, endocrine cells, pepsinogen-secreting chief cells, and rare tuft cells. In the antrum, LGR5⁺ cells in the gland base and SOX2⁺ cells in other gland regions differentiate almost exclusively into pit, endocrine, mucous (gland base) and rare tuft cells. Troy⁺ chief cells in the corpus and rare VIL1⁺ cells in the antrum can be recruited into a stem-cell role when the stomach mucosa is injured. Ant, antrum gland unit; Cor, corpus gland unit; Duo, duodenum; Eso, esophagus; Fst, forestomach; Pan, pancreas.

rare, have unclear functions and express chemosensory markers and characteristic apical microtubules. In addition to pit, endocrine and rare parietal cells in the antrum, cells located at the gland base secrete protective acidic mucins.

Each of these cell types is generated by stem and progenitor cells located in the isthmus of discrete gland units (Fig. 4). Radioactive labeling studies first revealed the dynamics of these granule-free cells in adult animals (Lee and Leblond, 1985). Subsequent analyses of chromosome patterns in XX-XY chimeric mice (Thompson et al., 1990) and of strain-specific antigens in C3H; BALB/c chimeric mice (Tatematsu et al., 1994) indicated that gastric glands are largely monoclonal, although 10–25% of glands remain polyclonal in adults (Nomura et al., 1998). Tracing an X-linked *lacZ* transgene after random X-chromosome inactivation in mice showed that glands begin as polyclonal units and rapidly become monoclonal in the first 3 weeks of life, a period that coincides with extensive gland fission (Nomura et al., 1998), whereby individual glands enlarge and subsequently produce two glands. Because both gland fission and the emergence of monoclonality occur more slowly thereafter, these processes are likely to be coupled. However, whether individual glands are derived from single progenitor cells or from multiple progenitors during development remains unclear. Moreover, analysis of mouse transgenes (Bjerknes and Cheng, 2002) and human mitochondrial DNA (McDonald et al., 2008) in the adult stomach provides divergent evidence for the presence of single or multiple stem cells within individual gastric glands.

Although the newborn mouse stomach mainly contains rudimentary glands, mucosal cells do express lineage-specific genes, indicating that the epithelium initiates differentiation late in gestation and continues to mature after birth (Keeley and Samuelson, 2010). Distinct transcriptional programs underlie the distinctive features of each epithelial lineage and gene targeting studies in mice have identified some of the TFs that are likely to be essential for emergence of discrete cell types. In the intestine, ATOH1 is a key lineage determinant whose absence eliminates all secretory cell types (Yang et al., 2001). By contrast, TF gene knockouts in the stomach typically reveal specific defects in individual non-endocrine cell types rather than global lineage losses, thus an analogous ‘master’ TF that specifies stomach cells remains undiscovered. Nonetheless, many TFs are expressed and control genes in specific stomach cell types. Examples include FOXQ1, which is restricted to pit cells and required for the expression of the gastric mucin *Muc5ac* (Verzi et al., 2008) and the basic-helix-loop-helix TF MIST1, which enables proper chief cell differentiation (Ramsey et al., 2007; Tian et al., 2010). XBP1 controls the latter process by inducing *Mist1* and expanding the rough endoplasmic reticulum (Huh et al., 2010a). In turn, MIST1 regulates mindbomb 1 (*Mib1*), which encodes a ubiquitin ligase that helps establish an apical secretory apparatus (Capoccia et al., 2013). Estrogen-related receptor gamma (*Esrrg*), which is highly expressed in parietal cells, controls specific genes including *Atp4b*, which is responsible for acid secretion (Alaynick et al., 2010). The Ets-domain TF SPDEF is essential for antral mucous cell differentiation (Horst et al., 2010), akin to its role in the maturation of intestinal goblet and Paneth cells (Gregorieff et al., 2009).

The specification of the various gastric endocrine cell populations is better understood. The stomach has five principal endocrine cell types – G cells (gastrin), D cells (somatostatin), enterochromaffin (EC) cells (serotonin), EC-like cells (histamine) and X/A cells (ghrelin) (Solcia et al., 2000) – and mouse gene knockout studies have provided insights into how each of these is specified (Fig. 5). The basic-helix-loop-helix TF gene *Ascl1* is required for all stomach endocrine lineages (Kokubu et al., 2008), whereas *Ngn3* and *Pax6* are necessary to produce both G and D cells, which probably act in a common progenitor (Larsson et al., 1998; Jenny et al., 2002; Lee et al., 2002). Further downstream, *Nkx6-3* and *Pdx1* are selectively required for G cells (Larsson et al., 1996; Choi et al., 2008); *Arx* is necessary for G cells and less-defined glucagon-expressing cells (Du et al., 2012); *Pax4* is essential for D cells (Larsson et al., 1998). Surprisingly, not all endocrine cells arise *de novo* in the stomach epithelium: a recent gene expression and lineage tracing study suggests that some corpus endocrine cells originate in bone marrow-derived mast cells (Li et al., 2014). Nevertheless, the resulting TF hierarchy (Fig. 5) has sturdy parallels with pancreatic and intestinal endocrine cell differentiation, although the basis for the activity of each TF remains unclear. In the simplest model, multipotent or unipotent endocrine progenitors selectively express individual TFs, which, in turn, activate particular genes. Because endocrine cell sub-types can differ only by a few gene products, including signature hormones, each TF could control a limited cistrome. Chromatin immunoprecipitation-sequencing (ChIP-Seq) analyses of TF binding will be useful to test this idea.

The development of stomach smooth muscle and the enteric nervous system

The smooth muscle of the stomach is thicker than that of other digestive organs, but the mechanisms of stomach-specific

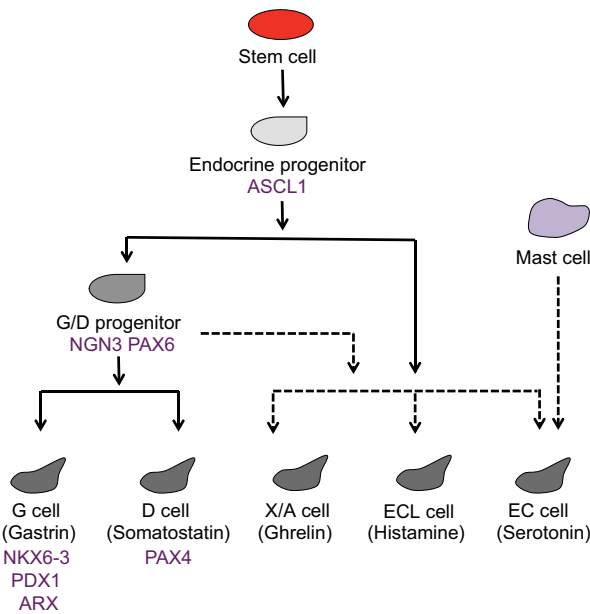


Fig. 5. Transcription factors implicated in stomach endocrine cell specification. The stomach contains five principal endocrine cell types: G cells (gastrin-producing), D cells (somatostatin-producing), enterochromaffin (EC) cells (serotonin-producing), EC-like cells (histamine-producing) and X/A cells (ghrelin-producing). *Ascl1* is expressed in all endocrine progenitors of the stomach during development and its deletion eliminates endocrine cells. Mice deficient for *Ngn3* or *Pax6* lack G and D cells, implying a common progenitor for these cell types. Further downstream, NKX6-3, PDX1 and ARX are required to produce G cells, whereas PAX4 is essential to produce D cells. Although NGN3⁺ endocrine progenitors can give rise to other cell types – X/A, ECL and EC cells – these cells are preserved in *Ngn3*-null mice, suggesting lack of a non-redundant requirement. Surprisingly, EC cells in the corpus seem to derive from non-epithelial mast cells.

myogenesis are not well understood. Hh signaling has a role in the differentiation of all gut smooth muscle: Hh inhibition impairs the differentiation and proliferation of myogenic progenitors, whereas excess Hh signaling expands the pool of progenitors (Ramalho-Santos et al., 2000; Mao et al., 2010) through unclear mechanisms. As noted above, forced expression of the stomach mesenchyme-specific TF BARX1 in intestinal mesenchyme converts intestinal smooth muscle into the stomach type; this occurs through robust proliferation of myogenic progenitors, which is likely to be mediated by intermediate TFs such as SIX2 (Jayewickreme and Shivdasani, 2015).

Specialized muscle cells in the pyloric sphincter integrate neuronal and hormonal signals to control the transit of food into the intestine (Ramkumar and Schulze, 2005). Studies in mouse and chick embryos have revealed the roles of certain TFs and intercellular signals – some of which also mediate other aspects of gastric development – in the specification and differentiation of this structure. *Barx1*-null mice, for example, lack a pylorus (Kim et al., 2005), possibly as a result of reduced *Bapx1* and/or *Six2* expression; the latter genes are expressed in the nascent pyloric sphincter, including – in the case of *Six2* – frog and chick embryos, and mice lacking either gene have pyloric defects (Self et al., 2009; Verzi et al., 2009). As first demonstrated in chick embryos, BMP signaling from the small intestine to the posterior stomach (GZ) mesenchyme triggers pyloric sphincter formation through expression of *Nkx2-5* and *Sox9* (Smith et al., 2000; Moniot et al., 2004; Theodosiou and Tabin, 2005). A detailed analysis of this region in mouse embryos recently revealed that *Nkx2-5* and *Gata3*

independently activate *Sox9* to promote differentiation of a dorsal fascicle of smooth muscle required for pyloric sphincter form and function (Udager et al., 2014). Along the stomach's lesser curvature, the sphincter is contiguous with superficial ligamentous cords that develop concomitantly with this dorsal fascicle; formation of these ligaments also depends on *Gata3* and *Nkx2-5* (Prakash et al., 2014). Stomach mesenchymal cells also give rise to intermuscular tendons. During this event, FGF signaling activates the basic-helix-loop-helix TF gene *Scleraxis* in selected cells primed for tendon differentiation; inhibition of *Scleraxis* impairs both tendon and smooth muscle development, revealing interdependency between these two cell types as they develop (Le Guen et al., 2009).

Gastric and enteric motility is regulated by the coordinated actions of smooth muscle, interstitial cells of Cajal and the enteric nervous system (Wallace and Burns, 2005), with additional input from certain hormones. *Kit* mutant mice lacking interstitial cells of Cajal have significantly attenuated excitatory and inhibitory enteric responses, revealing the importance of these cells in stomach muscle innervation (Beckett et al., 2002). The enteric nervous system (ENS) emerges from vagal neural crest cells that migrate early in development (Sasselli et al., 2012). Ret-GDNF signaling is critical in this chemoattractant-induced cell migration (Young et al., 2001; Natarajan et al., 2002), although it is unclear exactly how neural crest cells populate different regions of the gut tube. The ablation of vagal enteric neural crest cells in chick embryos recently revealed a novel role for the ENS in stomach patterning and smooth muscle development (Faure et al., 2015). This ablation led to sustained activation of BMP and Notch signaling in the stomach mesenchyme, with subsequently impaired myogenesis; both ENS ablation and ectopic Notch activation induced intestinal differentiation in the stomach. Although genetic proof of this unexpected ENS function is lacking in other species, these findings suggest that coordinated tissue differentiation in the stomach involves cells beyond the nascent epithelium and immediately adjacent mesenchyme.

Stomach stem cells and homeostasis

Lifelong self-renewal of the stomach epithelium relies on the activity of multipotent stem cells. Although recent studies have started to characterize the molecular properties of these cells, confusion arises from observations that candidate stem-cell markers such as LGR5 and SOX2 appear to localize to different cells. LGR5, a definitive marker of intestinal stem cells (Barker et al., 2007), is expressed in groups of cells at the base of glands in the antrum and gastric cardia, but not the corpus (Fig. 4). Similar to their intestinal counterparts, LGR5⁺ cells in the antrum display stem-cell activity (Barker et al., 2010) and respond to Notch signals (Demitrack et al., 2015), and their frequent symmetric cell divisions through neutral competition yield single dominant clones (Leushacke et al., 2013). SOX2 is expressed in gastric corpus and antral glands (Fig. 4), although not in a restricted gland zone (Arnold et al., 2011), and LGR5⁺ and SOX2⁺ cells seem to represent distinct populations, with limited spatial overlap, implying the existence of distinct stem cell populations. Moreover, intestinal crypts harbor additional, quiescent LGR5⁻ stem cells that become active in the event of epithelial damage (Clevers, 2013), and it is possible that an analogous population exists in the stomach. Indeed, rare antral cells expressing VIL1 (Fig. 4), which is normally expressed in the intestinal epithelial brush border, are quiescent for long periods but replicate when stimulated by a cytokine (Qiao et al., 2007). Notably, damage to the squamous epithelium adjoining the gastric cardia

induces cephalad migration of LGR5⁺ cells from this region, and the progeny of these cells produce columnar cells in the area of injured stratified epithelium (Wang et al., 2011; Quante et al., 2012). These findings raise the provocative idea that Barrett's esophagus (intestinalization of the squamous epithelium) might not represent bona fide metaplasia, but in fact is the outcome of mislocalized gastric stem cells (Wang et al., 2011; Quante et al., 2012). Although lineage tracing *in vivo* shows that SOX2⁺ cells in the corpus can generate all epithelial cell types for long periods, these cells are not found in the isthmus (Arnold et al., 2011) and markers specific to stem cells in the corpus isthmus have yet to be identified. Moreover, the developmental origins of gastric epithelial stem cells remain unclear and firmer characterization of the adult cells is necessary for further progress.

In addition to renewal from multipotent progenitors, stomach epithelial cells can also be replenished by de-differentiation of cells that appear to be terminally mature. For example, Notch signaling is active in stem cells in the isthmus and is required for their proliferation (Kim and Shivdasani, 2011), but ectopic Notch activation in parietal cells induces their de-differentiation into stem cells (Kim and Shivdasani, 2011). Similarly, differentiated Troy (also known as TNFRSF19)-positive chief cells (Fig. 4) represent a latent stem-cell pool, with epithelial injury inducing their de-differentiation (Stange et al., 2013). In addition, cells expressing the cholecystokinin 2 (CCK2) receptor overlap partially with LGR5^{low} antral cells and can convert into LGR5^{high} stem cells (Hayakawa et al., 2015). Together, these findings reveal considerable plasticity among stomach epithelial cells. Similar plasticity in the intestinal crypt has been attributed to a broadly permissive chromatin state that is present in LGR5⁺ stem cells as well as divergent progenitors (Kim et al., 2014). The stomach epithelial epigenome has not been examined, but might follow the same organizing principle, with chromatin in all cells broadly

primed to implement different transcriptional programs in response to specific TFs.

***In vitro* stomach culture systems**

Given their ability to self-renew, stomach and intestine stem cells are natural subjects for research in the field of regenerative medicine. Moreover, induced pluripotent stem cell (iPSC) technology has stimulated interest in inducing tissue regeneration and generating artificial organs *in vitro*. Much of the recent progress in this context has built upon knowledge about the sequence of signals and events during development of the alimentary canal and on understanding cellular relationships and requirements. Using this knowledge, four independent approaches to generate stomach tissue *in vitro* – using iPSCs, embryonic stem cells (ESCs) or adult stem cells as a starting point – have been fruitful to date (Fig. 6).

Starting with various human pluripotent cells, Wells and colleagues modulated the signaling pathways that control endoderm development with temporal specificity to generate intact stomach tissues that contain both epithelial and sub-epithelial elements. After differentiating pluripotent human cells into definitive endoderm, they sequentially activated Wnt and FGF signaling to initiate tube morphogenesis, inhibited BMP to induce SOX2, and finally activated RA signaling to posteriorize the resulting stomach; this approach culminated in antral differentiation *in vitro* (McCracken et al., 2014). Adopting an approach that was similar in concept but quite different in the details, Noguchi and colleagues built on observations that Hh activity in the developing stomach is high (Ramalho-Santos et al., 2000), whereas Wnt signaling is actively suppressed (Kim et al., 2005). Their recapitulation of these pathway activities in mouse ESCs, followed by *Barx1* activation in mesenchymal cells, yielded stomach organoids that resemble either the antrum or corpus, with the latter containing mature parietal and chief cells (Noguchi et al.,

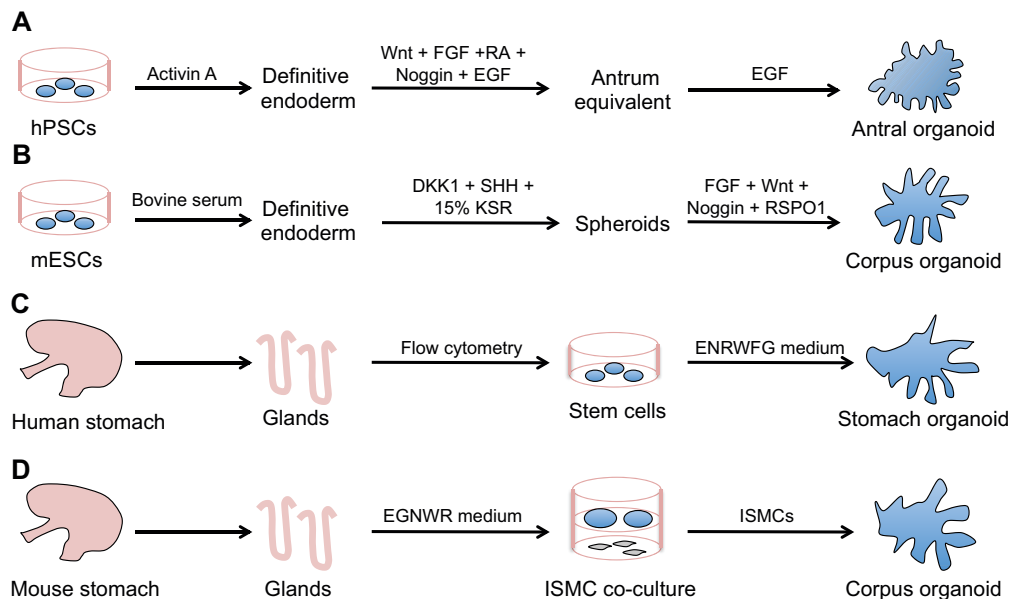


Fig. 6. Approaches to generate stomach organoid cultures *in vitro*. (A) After promoting the differentiation of pluripotent human stem cells (iPSCs or ESCs) to definitive endoderm with Activin A, antral organoids are established by further treatment with Wnt, FGF4, RA, Noggin and EGF (McCracken et al., 2014). (B) After induction of definitive endoderm in murine ESCs, DKK1, SHH and knockout serum replacement (KSR) are added to small spheroids, followed by 3D culture in medium containing FGF10, WNT3A, Noggin and RSPO1 to promote corpus organoid differentiation (Noguchi et al., 2015). (C) Single human gastric epithelial cells, isolated by fluorescent cell sorting, are exposed to EGF, Noggin, RSPO1, Wnt, FGF10 and gastrin (ENRWFG), followed by removal of Wnt, to induce stomach organoids (Bartfeld et al., 2015). (D) Isolated mouse stomach glands are cultured in EGNWR medium, followed by co-culture with immortalized stomach mesenchymal cells (ISMCs), to induce corpus organoids (Schumacher et al., 2015).

2015). Other groups have used native epithelial cells as the starting material for *ex vivo* tissue expansion. Clevers and colleagues isolated gastric glands from human corpus surgeries and used single stem cells from these glands to culture organoids (Bartfeld et al., 2015). Although these structures lacked parietal cells, perhaps because culture conditions were not ideal for this purpose, they did contain the four other cell types for long periods (Bartfeld et al., 2015). Finally, Zavros and colleagues developed two distinct approaches for stomach organoid cultures: one expands native stem cells, whereas the other relies on the co-culture of gastric epithelium with immortalized mouse fetal stomach mesenchymal cells to generate mature stomach cell types (Schumacher et al., 2015).

Beyond the application of these advances to regenerative therapy, which remains a distant prospect, stomach organoid cultures have immediate value in studying the pathogenesis of stomach disorders and perhaps also in high-throughput screens. For example, such organoid cultures have been used to examine how the *H. pylori* bacterium affects gastric epithelial cells. *H. pylori* colonizes the antral mucosa in nearly 50% of humans, inducing chronic tissue damage (De Falco et al., 2015) and hence elevating the risk for gastritis, peptic ulcers and cancer. *H. pylori* activates NF- κ B-mediated inflammation in gastric epithelial cells, eliciting the chemokine interleukin-8 (Keates et al., 1997) and its virulence factor CagA (also known as S100A8) forms a complex with the MET receptor tyrosine kinase, activating epithelial proliferation (Peek et al., 1997; Churin et al., 2003). These aspects of pathobiology have successfully been reproduced in antral organoid cultures derived from human ESCs (McCracken et al., 2014) or primary human corpus specimens (Bartfeld et al., 2015). Mouse organoid cultures have also been used to assess parietal cell function and repair following cell damage induced by a two-photon laser (Schumacher et al., 2015) and to replicate features of Menetriere disease (Noguchi et al., 2015), which is a rare premalignant disease of the stomach. These advances emphasize the value of insights from developmental biology in tissue engineering and *in vitro* disease modeling.

Common congenital and acquired adult stomach disorders

A refined understanding of organ development can shed equally useful light on birth defects and acquired disorders that affect the stomach. Among the congenital disorders that represent aberrant stomach development, infantile hypertrophic pyloric stenosis (pyloric stenosis) is the most common, with an incidence of 2–4 cases per 1000 live births. The condition is caused by muscle hypertrophy, which narrows the pyloric canal and creates functional gastric outlet obstruction (Peeters et al., 2012). Pyloric stenosis is in fact a complex disorder influenced by genetic and environmental factors, including maternal smoking and alcohol use. The implication of common variants near *MBNLI* and *NKX2-5* in a genome-wide association study (Feenstra et al., 2012) is noteworthy because *Nkx2-5* is expressed specifically in the developing pyloric sphincter and is necessary for its proper formation in chick and mouse embryos (Smith et al., 2000; Theodosiou and Tabin, 2005; Udager et al., 2014). Nitric oxide deficiency (Vanderwinden et al., 1992; Huang et al., 1993) and defects in the ENS (Guarino et al., 2000) or interstitial cells of Cajal (Vanderwinden and Rumessen, 1999) are also associated with pyloric stenosis and are likely to affect synchronized muscle contraction. Gastric outlet obstruction can alternatively reflect the rare congenital condition of pyloric atresia, which can occur in isolation or together with either esophageal and/or duodenal atresia or seemingly unrelated conditions such as epidermolysis bullosa and congenital heart

disease. Pyloric atresia is associated with mutations in several genes involved in the formation of hemidesmosomes (Vidal et al., 1995; Ruzzi et al., 1997; Pfendner and Uitto, 2005), hinting at defective cell adhesion as a root cause.

Certain signals used during stomach development seem to remain pertinent in adult gastric function and disease. For instance, *Shh* is expressed in adult parietal cells, where its loss leads to excess gastrin production and Wnt-responsive mucosal hyperproliferation (Xiao et al., 2010; Feng et al., 2014). BMP signaling also restrains stomach epithelial cell proliferation in adult mice, as indicated by the effects of deleting the BMPRI1A receptor or overexpressing *Noggin* (Bleuming et al., 2007; Huh et al., 2010b; Shinohara et al., 2010). Wnt signaling is transiently high in the forestomach early in development, attenuated after stomach specification (Kim et al., 2005) and appears again in the base of adult antral glands, which express LGR5 and other Wnt target genes (Barker et al., 2010). Wnt requirements in this setting are unclear, but it has been shown that Wnt signaling is activated in up to 30% of human gastric cancers and that *Apc^{Min}* mice develop antral adenomas (Clements et al., 2002; Tomita et al., 2007). A careful balance of the various cell types generated during stomach development also appears to be pertinent for adult gastric function. Spasmolytic polypeptide expressing metaplasia (SPEM) and other inflammatory gastric conditions, for example, are often accompanied by parietal cell loss and abnormal chief cell differentiation (Goldenring et al., 2010). The parietal cell loss leads to defects in epithelial homeostasis, inducing transdifferentiation of chief cells to SPEM (Li et al., 1996; Nam et al., 2010). Accumulating data indicate that intestinal metaplasia arises from SPEM, highlighting the significance of proper lineage differentiation (Yoshizawa et al., 2007; Nam et al., 2009; Goldenring et al., 2010). It is unclear whether these effects on two or more cell types are independent or reflect the targeting of a common progenitor. Supporting the latter possibility, occasional chief cells are labeled in parietal cell-specific *Atp4b-Cre* mice (Kim and Shivdasani, 2011).

In light of their seminal roles in stomach development, it is possible that the same TFs that control stomach development have important roles in gastric disease. Gastric adenocarcinoma develops through a sequence of aberrant states, including atrophic gastritis with foveolar hyperplasia or SPEM and intestinal metaplasia (Correa, 1988; Goldenring et al., 2010). Although the developmental framework for these transitions has been elusive, some studies have implicated developmental TFs and signals in mediating the changes. On average, Notch receptors, ligands and the target gene *Hes1* are expressed at higher levels in cancerous epithelium compared with normal stomach epithelium (Du et al., 2014) and, in support of a pathogenic role, prolonged activation of Notch in the mouse epithelium induces adenomas in the corpus (Kim and Shivdasani, 2011) and antrum (Demitrack et al., 2015). Mice with parietal cell-specific *Shh* deletion develop foveolar hyperplasia (Xiao et al., 2010), whereas loss of *SHH* in humans correlates with atrophic gastritis and intestinal metaplasia (Shiotani et al., 2005). Ectopic expression of the intestine-restricted TFs CDX1 or CDX2 in the murine stomach is sufficient to induce intestinal features (Silberg et al., 2002; Mutoh et al., 2004a) and aged CDX2-overexpressing mice even develop gastric polyps (Mutoh et al., 2004b). Although these findings might be interpreted to reflect roles for SHH and CDX-family TFs in the adult disease sequence, it should be noted that loss of SHH and ectopic CDX expression in these studies began in the embryo, so it is unclear whether these are causal factors or simply markers of intestinal metaplasia. The role of SOX2 is also confusing, in part because its

expression is reduced in some gastric cancers and increased in others. Adding to the uncertainty, *SOX2* overexpression in some gastric cancer lines arrests cell replication and induces apoptosis (Otsubo et al., 2008) but inhibition of *SOX2* has similar effects in the AZ-521 human gastric cancer cell line (Hutz et al., 2014). The significance of many of these associations is unclear, leaving much to learn about the relationship between developmental regulation and adult gastric disorders.

Conclusions

As highlighted above, certain TFs and intercellular signals are utilized repeatedly in distinct contexts and locations during stomach development. A detailed understanding of these determinants will no doubt inform current paths toward tissue and disease modeling. A second theme in stomach development is the tight spatial and temporal control of signal exchange between the epithelium and adjoining mesenchyme. An important goal is to understand the basis for these coordinated tissue interactions and how ubiquitous signals elicit exquisitely specific responses in different contexts. The characterization of stomach cell epigenomes and TF activities will also help to reveal the basis for the stable and malleable cell states present during stomach development and in adults. Finally, various lines of evidence suggest the presence of multiple stem cell pools in the stomach epithelium, but the relationships between these populations and their respective properties and developmental origins remain obscure. Current efforts toward intravital imaging, the identification of additional specific markers, and refined lineage tracing should shed useful light on these questions and on stomach cell plasticity and disease states.

Competing interests

The authors declare no competing or financial interests.

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