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Mechanisms of embryonic stomach development

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Abstract

The stomach is a digestive organ that has important roles in human physiology and pathophysiology. The developmental origin of the stomach is the embryonic foregut, which also gives rise a number of other structures. There are several signaling pathways and transcription factors that are known to regulate stomach development at different stages, including foregut patterning, stomach specification, and gastric regionalization. These developmental events have important implications in later homeostasis and disease in the adult stomach. Here we will review the literature that has shaped our current understanding of the molecular mechanisms that coordinate gastric organogenesis. Further we will discuss how developmental paradigms have guided recent efforts to differentiate stomach tissue from pluripotent stem cells.

Keywords

endoderm; foregut; stomach; corpus; fundus; antrum

1. INTRODUCTION

The stomach is an evolutionarily diverse structure that has numerous functions including digestion of food, immune defense, and hormonal regulation of metabolic homeostasis. Depending on their unique dietary needs and habits, vertebrates have adapted variations in their structural and histological organization of the stomach [1]. In humans, the gastric mucosa entirely comprises a glandular, columnar epithelium, and it is regionalized into two distinct compartments containing specialized cell types that work in a complementary fashion. The more proximal region of the stomach contains fundic glands that contain acid-secreting parietal cells, enzyme-producing chief cells, and protective mucus-forming cells, whereas the distal region of the stomach, the antrum, consists largely of mucous cells and

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endocrine cells including G-cells that secrete gastrin. In addition to these, however, mice have a large forestomach in the anterior, which consists of a stratified squamous epithelium similar to that of the esophagus.

Regardless of its ultimate composition, the mechanisms that direct early embryonic development of the stomach appear to be conserved across vertebrate species. While the species-specific differences in architecture can sometimes hamper our ability to directly translate findings from animals to humans, we have learned a great deal about stomach development from model organisms including mouse, chick, frog, and fish. Additionally human pluripotent stem cell (hPSC)-derived endoderm has recently proven to be a valuable *in vitro* model system for interrogating the dynamic processes of foregut and stomach development. In this review, we will summarize the early developmental mechanisms of stomach development, focusing on the signaling pathways and transcriptional regulators that control endoderm patterning, gastric specification, stomach regionalization, and morphogenesis. While the later control of gastric stem cell behavior and homeostasis likely has parallels to early developmental processes, this literature has been reviewed recently [1–3] and will not be discussed here.

2. SPECIFICATION OF GASTRIC LINEAGE

The embryonic stomach derives from posterior foregut, and it develops amid a number of neighboring tissues including the esophagus, intestine, liver, gallbladder and pancreas. As with development of all endoderm organs, a complex array of epithelial-mesenchymal interactions is responsible for promoting gastric fate. In both rodent and chick embryos, presumptive gastric mesoderm is required to specify a gastric fate in the nearby endoderm, and it can re-specify endoderm taken from non-gastric regions of the gut [4,5]; however, the responding endoderm is only competent to respond to gastric-inducing signals during a short temporal window in early development [6], suggesting that gastric specification is a temporally and spatially dynamic process. The work of several groups has led to the elucidation of signaling pathway and transcription factor networks that govern some of the early stages stomach development.

2.1 Patterning posterior foregut endoderm

Following gastrulation a series of instructive signals patterns nascent endoderm resulting in a three-dimensional gut tube that is roughly divided into anterior and posterior domains (Reviewed in [7]). The posterior region, or hindgut, expresses the homeodomain transcription factor *Cdx2* [8,9], and it gives rise to the small and large intestines. The anterior endoderm is the foregut, which expresses the HMG box transcription factor *Sox2* [9]. The signals responsible for this early anterior-posterior patterning have been elucidated in numerous model organisms, and they include WNT, FGF, and BMP [10–13]. Interestingly, all of these pathways have been shown to have a posteriorizing effect on early endoderm. In the nascent foregut, expression of *Sox2* is negatively regulated by BMP signaling [14–16]. Moreover, repression of BMP signaling occurs throughout early stages of esophagus and stomach development [17], suggesting that these organ fates require a low-BMP state. Although WNT, FGF, and BMP have direct effects on endoderm [18–20], they

only have patterning activity in the presence of mesoderm, suggesting that multiple signals might act synergistically to coordinate endoderm patterning. Indeed, activity of all three pathways is required to differentiate hPSCs into Cdx2-positive hindgut tissue [16,21].

Shortly after its formation, the Sox2-positive foregut tube is segregated into a number of primitive organ domains. The anterior foregut, in the pharyngeal region of the embryo, forms the oropharynx, thyroid, thymus and parathyroid glands. More posterior portions of the foregut differentiate into the epithelial lining of the esophagus and glandular stomach, as well as budding organs including lungs, liver, pancreas, and extrahepatic biliary system. The ability to give rise to such a diversity of organ lineages implies that the foregut itself might be patterned into sub-domains that have distinct developmental potentials. However this stage of development is brief and difficult to access experimentally, so there are limited data that address this concept of "foregut patterning." Evidence to support this model comes via the regionalized expression of several transcription factors that are expressed in the posterior foregut prior to organ specification, including *Hnf1β*, *Hnf6*, and *Prox1* [9,22–24]. In particular, Hnf1β appears to be a master regulator of posterior foregut development as its deletion leads to aberrant specification of the pancreas, liver, and glandular stomach [24,25].

On key regulator of posterior foregut development is retinoic acid (RA), which has numerous roles in pattern formation in embryonic development (reviewed in [26]). *Raldh2*, the primary synthetic enzyme in the pathway, is expressed in posterior mesodermal tissues surrounding the distal foregut and hindgut [27], implicating RA as an important foregut patterning molecule. Early RA activity is required for specification of several posterior foregut derivatives including the pancreas [28,29] and lung [15], and it represses the development of anterior foregut lineages such as the thyroid [30]. The glandular hindstomach fails to form in mice lacking *Raldh2* [31], providing further evidence for a role for RA in posterior foregut development. Consistent with this, application of exogenous RA is critical for the *in vitro* differentiation of human gastric tissues from hPSCs [16]. In these models RA promotes expression of *HNF1β*, suggesting that a RA-Hnf1β regulatory network might control early foregut patterning to set up competence for specification into posterior foregut lineages.

Aside from the few studies described above, there is a paucity of information known regarding the molecular pathways that govern formation of the posterior foregut. Reduction in Activin receptor signaling leads to similar gastric phenotypes as observed in *Raldh2* mutants [32], but the TGFβ pathway has not been more thoroughly investigated for its role in stomach development. With there being high interest in generating fully functional foregut organ tissues from hPSCs, it is essential to better delineate the mechanisms that segregate early foregut endoderm into its derivatives.

2.2 Defining the anterior gastric boundary

After initial patterning, the posterior foregut is further divided into distinct organ lineages. Again, the molecular mechanisms that precisely drive these events are poorly understood. Instead, much of our knowledge is derived from studies focusing on the establishment of organ-organ boundaries, i.e. binary fate decisions. The presumptive gastric epithelium forms adjacent to the esophagus, small intestine, pancreas, and liver; thus, stomach specification in

the posterior foregut involves processes that exclude or repress each of these other organ fates.

The process of establishing the gastro-esophageal boundary is likely to be different in mice than it is in humans because of the divergent anatomy between mammalian species. Because the mouse has a forestomach that resembles the esophagus, the gastric columnar epithelium only begins at the convergence of the forestomach and hindstomach. In contrast, the squamo-columnar junction in humans occurs at the organ boundary between the stomach and esophagus. At the molecular level, it seems that gene expression boundaries follow the squamo-columnar junction pattern rather in mice rather than the gastro-esophageal junction. Moreover, the embryologic precursors of the esophagus and forestomach display similar developmental potential. For example, ectopic activation of β-catenin is sufficient to convert presumptive esophageal progenitors into the *Nkx2-1*-positive respiratory lineage, and it has a similar effect on anterior but not posterior stomach epithelium [33,34]. Therefore, it seems most likely that the forestomach-hindstomach boundary in rodent embryos is analogous to the gastro-esophageal boundary in human development.

Hedgehog pathway ligands are generally expressed by the endodermal lining of the developing gut tube and they primarily signal to the adjacent mesodermal tissue. Most of the early endoderm in mice expresses *Sonic hedgehog (Shh)*, but the presumptive glandular hindstomach epithelium specifically expresses *Indian hedgehog* (*Ihh*; [35]). Thus, there is a *Shh-Ihh* expression boundary at the junction of the forestomach and hindstomach. There are two lines of evidence that the gastric mesenchyme plays an essential role in establishing this junction. First, deletion of the mesenchymal factor *Hoxa5* disrupts the normal *Shh-Ihh* expression pattern leading to defective hindstomach differentiation [36]. Second, deletion of *Shh*, which is believed to only signal directly to the mesenchyme [37], also disrupts the forestomach-hindstomach boundary. In both Hoxa5 and Shh mutants [38], the hindstomach also adopts some intestinal features. These data suggest a model whereby Hoxa5, acting through an unknown paracrine factor, regulates epithelial Hh ligand expression, which in turn regulate mesenchymal growth factors that further reinforce the forestomach-hindstomach junction. Thus, the extrinsic signaling mechanisms that direct epithelial cell fate decisions at the anterior gastric boundary remain largely unknown.

A gastric-specific transcriptional “master regulator” has not yet been identified during embryonic stages. However there are several broadly expressed factors that contribute to establishing the esophageal-gastric boundary. The zinc finger transcription factor *Gata4* is robustly expressed in the hindstomach and completely excluded from the forestomach. *Gata4*-null cells in chimeric embryos do not contribute to the glandular epithelium of the stomach, and deletion studies demonstrated that Gata4 interactions with its transcriptional co-activators FOG1/2 are required for normal gastric development [39,40]. Further, deletion of *Gata4* from the already-specified distal stomach causes cell-autonomous migration of cells from the hindstomach into the forestomach [41]. A related factor, *Gata6*, has a similar localization pattern [42] but has not been investigated for a role in stomach development. Thus, Gata4 remains one of the very few genes known to be required for stomach specification. The early foregut gene *Sox2* remains expressed in both the esophagus and stomach throughout much of development. While it is required for proper differentiation of

the squamous epithelia of the foregut [43], glandular stomach differentiation appears unperturbed in Sox2 hypomorphs. Though it would be an important finding that Sox2 is dispensable for gastric development, complete loss-of function analyses have not yet been performed.

2.3 Defining the posterior gastric boundary

The gastro-intestinal junction is established as early as E8.5 in the mouse embryo, as it is identified as the *Sox2*-*Cdx2* expression boundary. Although a small number of cells co-express these factors in the early endoderm [9], the border is sharpened and refined over developmental time [44]. These transcription factors are important regulators of foregut and hindgut fates, respectively. Ectopic activation of *Sox2* in the hindgut suppresses the intestinal phenotype and induces features of the glandular stomach, but it does not repress *Cdx2* itself [45]. Similarly, forced expression of *Cdx2* in previously specified developing gastric epithelium is sufficient to induce intestinal metaplasia [46] but not down-regulation of *Sox2* [47]. These findings that Sox2 and Cdx2 are not mutually repressive are interesting, given that they have dominant effects on gastric and intestinal differentiation. Moreover, it is also fascinating that *Cdx2* loss-of-function has different effects depending on the timing of deletion. Early loss of *Cdx2* (via *Foxa3*-Cre) leads to impaired intestinal differentiation, but the intestine adopts an esophageal, rather than gastric, phenotype [48]. In this model, the gastric boundary is not impacted. Conversely, later deletion of *Cdx2* with *Villin*-CreER produces pyloric-like gastric glands in the intestine [49]. Taken together, these studies have revealed that Sox2 and Cdx2 have key functions in establishing the gastro-intestinal boundary and maintaining organ fate. However, the findings also suggest that there must be unidentified factors that contribute to determining gastric and intestinal fates.

Early formation of the gastro-intestinal boundary is orchestrated by a mesenchymal-epithelial network that was elucidated in a key series of papers on the transcription factor *Barx1* [50,51]. This factor is specifically expressed in the presumptive stomach mesenchyme, and it promotes expression of secreted WNT signaling antagonists (sFRPs) in this region. This Barx1-mediated WNT inhibition is required to suppress intestinal transformation in the presumptive gastric epithelium. While WNT/β-catenin activation is able promote hindgut gene expression in several model systems including frog, mouse, and human endoderm [10,19,21], ectopic induction of the Barx1-sFRP cassette in hindgut mesenchyme does not impact differentiation of the intestinal epithelium [52]. This discrepancy could be explained by either timing of WNT signaling repression or the existence of other signaling pathways that regulate this fate decision. One example is the BMP signaling pathway, which in human endoderm models *in vitro* is necessary for intestinal specification, and it must be inactive during early foregut development [16]. Along similar lines, BMP is sufficient to repress *Sox2* expression in the foregut [14], and BMP activity is accordingly low in the embryonic stomach and esophagus. Taken together, these studies indicate that multiple signaling pathways, including at least WNT and BMP, act in combination to establish organ boundaries at the foregut-hindgut junction.

2.4 Gene expression boundaries between stomach and accessory organs

In addition to the competing lineages along the longitudinal axis of the alimentary canal, early gastric progenitors must also be specified apart from the budding organs of the posterior foregut – the pancreas and liver. Again, there are transcription factor boundaries that distinguish between these early organs domains. While the pancreatic factor *Pdx1* is also expressed in the distal stomach and proximal intestine epithelia, the bHLH factor *Ptf1a* is highly specific to the pancreas at E9.5 [53,54]. As an example of the early plasticity of posterior foregut endoderm, ectopic *Ptf1a* expression is sufficient to induce pancreatic fate in gastric progenitors [55,56]. Deletion of *Hes1*, another bHLH factor, also leads to ectopic pancreatic differentiation along the greater curvature of the stomach [57], suggesting that *Hes1* represses pancreatic fate in the presumptive stomach. Although Hes1 often functions downstream of Notch signaling, gastric specification defects are not observed in other Notch pathway mutants [58]. Therefore Hes1 might act independent of Notch, but neither its function nor that of the Notch pathway has been further explored in the developing stomach.

As described previously, the stomach and liver develop adjacent to each other and express a number of common markers, such as *Hnf1β*. Despite their proximity in the foregut, little is known regarding the mechanisms that segregate early gastric and hepatic lineages, except that BMP and FGF activation promote liver specification [59,60]. Thus absence of these factors is thus likely permissive for stomach development, again emphasizes the inhibitory role of BMP in stomach specification. One interesting observation is that while the lateral (presumptive gastric) foregut endoderm at E9.5 expresses Gata4, the budding hepatic diverticulum is *Gata4*-negative [61]. These data again imply a potentially important role for Gata4 in foregut patterning and lineage segregation, but early endoderm-specific knockouts have not yet been described.

The key signaling pathways and transcription factors required for posterior foregut patterning and gastric specification are summarized in Figure 1.

3. PATTERNING THE EMBRYONIC STOMACH

Once specified, gastric progenitors are further divided along the rostro-caudal axis. The proximal region forms the epithelium of the corpus and fundus (and additionally the forestomach in mice), while the distal portion develops into the antral stomach. During embryonic stages, one of the few known molecular differences between these gastric domains is *Pdx1*, which is specifically localized to the presumptive antral region at E10.5 [62]. Surprisingly, the role for Pdx1 in stomach patterning has not been investigated, outside of its known requirement for the differentiation of antral G-cells at later stages [63]. *Pdx1* mutant embryos do not exhibit gross or obvious defects in stomach morphology [64], but it would be interesting to see whether this transcription factor impacts gastric differentiation more generally. The transcription factor *Nkx6.3* is also expressed in an antrum-specific fashion, but seems to only be required for G-cell specification within the antrum [65]. In order to more fully understand the mechanisms of stomach pattern formation, it is imperative to identify additional genes that exhibit region-specific expression profiles.

Predictably, mesenchymal-epithelial interactions appear to play an important role in gastric patterning. Bapx1 is a transcription factor expressed in mesenchyme surrounding the distal stomach and proximal intestine [66]. When it is deleted, the antral segment of the stomach is reduced and parietal cells are found near the gastro-intestinal junction. The signaling mechanism underlying this phenomenon has not been identified in rodents. However in the avian stomach, Bapx1 represses the expression of *Wnt5a* and *Bmp4* [67], suggesting that WNT and/or BMP signaling may play a role in stomach patterning. It has been observed that canonical WNT activity is restricted to the rostral embryonic stomach and excluded from the presumptive antrum [50,68,69]. Following up this observation, it was shown that deletion of the canonical WNT effector *Ctnnb1* in the epithelium led to ectopic *Pdx1* expression and subsequent antralization of the proximal stomach. In these mice, loss of *Ctnnb1* resulted in disruption of fundic-type cytodifferentiation in a cell autonomous manner. These data support a model in which WNT/β-catenin signaling can toggle between cell fates in the developing glandular stomach. High WNT activity is required for differentiation of the corpus/fundus region, while suppression of the pathway permits specification of the antral domain (summarized in Figure 2). Deletion in these animals largely occurred prior to gastric patterning, so it is yet unclear whether WNT/β-catenin manipulation can impact gastric fate after initial patterning has already occurred. This principle was further applied to modulate cell fate during the *in vitro* differentiation of PSCs into human gastric organoids (hGOs). In the absence of WNT signaling hGOs contain *Pdx1*-positive antral epithelium, but early WNT stimulation induces fundic-type hGOs that contain parietal and chief cells [70].

The newer data that support a role for WNT/β-catenin signaling in formation of the corpus/fundus appear incompatible with previous models in which WNT signaling must be repressed during stomach development. There are several potential explanations that could resolve this discrepancy. Time is an important variable in embryonic development, and it is thus possible that early WNT promotes intestinal specification while a later WNT signal regulates gastric patterning. Further, the epithelial response to WNT could be modulated by activity of other pathways such as BMP, as described above. Regardless, more work is necessary to address these questions and identify how gastric fate is determined.

4. MORPHOGENESIS OF THE GASTRIC EPITHELIUM

The early foregut epithelium is a simple cuboidal lining that lacks architectural complexity. Following gastric specification and patterning, the mucosa transitions through a pseudostratified stage prior to adopting the tall columnar morphology that is found in the adult organ, similar to events observed in the intestine [71]. Accompanying these cell shape changes, the epithelium undergoes folding and glandular morphogenesis that progressively develops through postnatal stages, resulting in the highly organized, complex structure found in the mature stomach. The precise mechanisms that coordinate these morphogenetic events are not fully understood, but several embryonic signaling molecules are known to play a role.

Similar to its role in regulating cell fate decisions, the gastric mesenchyme is essential for proper growth and morphogenesis of the stomach. Epithelial-derived Hh ligands sustain mesenchymal cell survival and proliferation, and simultaneous deletion of *Shh* and *Ihh* thus

lead to severely stunted stomach and intestinal growth [72]. Despite their aberrant morphogenesis, proper gastric specification and patterning are maintained in these mutants. Downstream of the epithelial Hh signal, *FoxF* and *FoxL* transcription factors mediate a complex signaling network in the associated mesenchyme [73,74]. An important mesenchyme-derived growth factor is *Fgf10*, which signals to the epithelium through the *Fgfr2* receptor throughout the later stages of development. Loss of either molecule results in reduced proliferation and defective glandular morphogenesis in the gastric epithelium [75,76]. Conversely, overexpression of *Fgf10* leads to glandular hyperplasia and impaired cytodifferentiation [77]. Gland formation in the avian proventriculus is also regulated by BMP, WNT5a, Notch, and Shh [78–81]. In mouse, it was shown that *Wnt5a* activates the WNT/PCP pathway, which is essential for maintaining epithelial cell polarity, morphogenesis, and growth and elongation of the anterior portion of the stomach [69]. Further, canonical WNT signaling contributes to stomach growth as epithelial deletion of *Ctnnb1* leads to a dramatic reduction in stomach size [70], albeit downstream of yet unidentified WNT ligands. Finally, EGF is another important epithelial mitogen required for normal proliferation and growth of the rodent mucosa [82,83], and ectopic EGF is required for in vitro differentiation of embryonic gastric structures [16].

5. SUMMARY

Overall, a number of developmental signaling pathways are used reiteratively during stomach development to regulate endoderm patterning, gastric specification, stomach regionalization, and morphogenesis. Included in this group are WNT, Bmp, Fgf, and RA, and together they precisely coordinate expression of transcription factors that determine cell identity. Our current understanding of the transcriptional regulators of stomach development is lacking, and it is imperative for future work to further elucidate the signaling paradigms underlying this process. Nevertheless, use of known embryologic mechanisms, as well as newly discovered principles, has recently led development of protocols for the *de novo* differentiation of gastric tissue from PSCs [16,70,84]. These systems, in turn, should prove to be effective models for studying gastric development and disease. For example, there are currently no available data on the epigenetic control of stomach development, and *in vitro* tissues are well suited for epigenetic studies.

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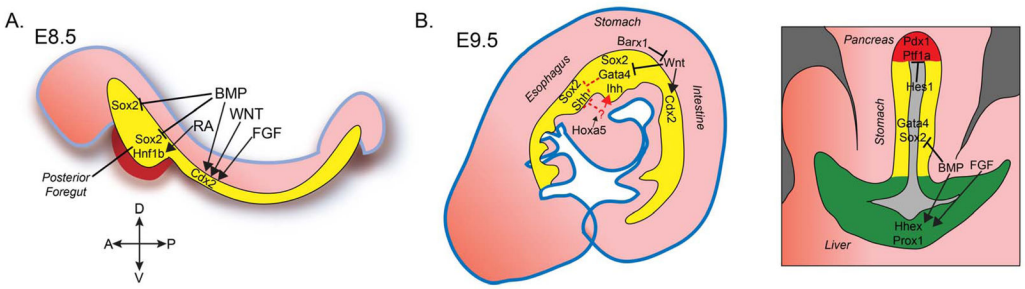

Figure 1. Schematic overview of developmental mechanisms leading to stomach specification

A. Patterning of posterior foregut endoderm. During endoderm patterning, which occurs around E8.5 in the mouse, WNT, Bmp, and Fgf signals promote hindgut (Cdx2) gene expression. BMP represses the foregut marker Sox2. RA activity is required for specification of the posterior region of the foregut, where one of its target genes is Hnf1β. B. Sagittal and transverse representations of lineage restriction and gastric specification in the mouse.

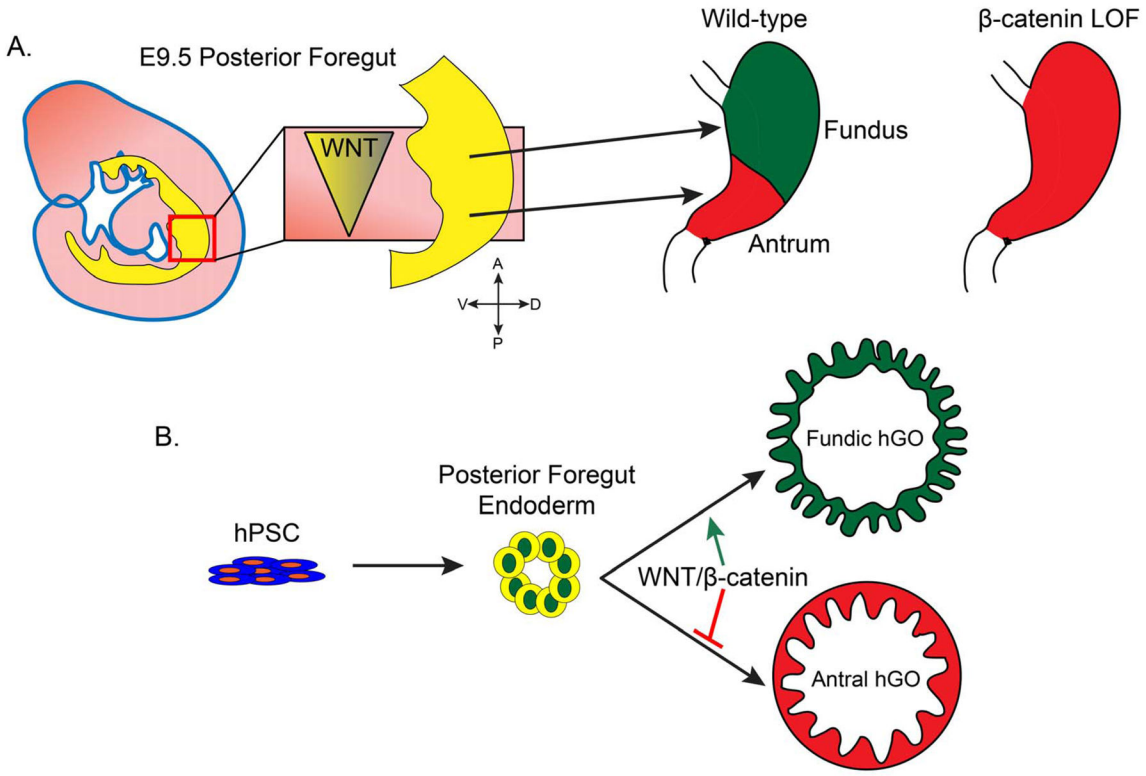

Figure 2. Summary of WNT-mediated gastric patterning mechanisms *in vivo* and *in vitro*

A. WNT/β-catenin signaling promotes fundus specification from the pool of gastric progenitor cells during E9–10. B. WNT activation promotes generation of fundic human gastric organoids (hGOs), while repressing antral specification, from hPSC-derived posterior foregut spheroids.