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Review

Mechanisms of regulation of normal and metaplastic intestinal differentiation

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Summary. The gastrointestinal tract is an organized structure originating from the three embryonic germ layers: endoderm, mesoderm and ectoderm. Morphological changes that accompany its formation are relatively well known, although the underlying molecular mechanisms are still poorly defined. Intestinal metaplasia, resulting from an epithelial trans-differentiation process, is considered a precursor lesion of gastric adenocarcinoma, a malignancy with serious consequences in terms of morbidity and mortality worldwide. Similarly to gastrointestinal embryonic development, molecular changes involved in the development of this lesion that recapitulate the intestinal development, out of time and space, are also widely unknown.

In this review we present, briefly, the process of formation of the digestive tract, from its embryonic age to adulthood, with emphasis on anterior-posterior patterning and on molecular mechanisms that may play an important role. In addition, we try to establish a parallel and understand what mechanisms can, through their deregulation, originate the metaplastic lesion.

Cdx genes appear to be the main regulators of normal intestinal differentiation and also to be largely involved in the metaplastic epithelial transdifferentiation process. However, control of gene expression both during intestinal development and in intestinal metaplasia is complex and seems to depend on several transcription factors. More extensive studies about the mechanisms underlying intestinal metaplasia are needed if we aim to prevent neoplasia development and all its negative consequences in persons at risk.

Key words: Gastrointestinal differentiation, Gastrointestinal development, Intestinal metaplasia, CDX2, Transcription factors

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Introduction

The gastrointestinal (GI) tract is a long tubular system encompassing the esophagus, stomach, small bowel and colon. It originates at the endoderm and mesoderm and is innervated by cells originating from the third germ layer, the ectoderm. It is a superiorly organized structure that serves numerous and important functions, including nutrient digestion and absorption, endocrine and immune functions (Spence et al., 2011a,b).

Intestinal metaplasia (IM) of the stomach is a preneoplastic lesion resulting from a process of transdifferentiation of the gastric epithelium into an intestinal-type epithelium. This condition takes part of a sequence of changes in the gastric mucosa, triggered in the majority of cases by the infection with *Helicobacter pylori*, which may ultimately give rise to gastric cancer (Mesquita et al., 2006; Gutierrez-Gonzalez and Wright, 2008; Busuttil and Boussioutas, 2009; Kang et al., 2011). Although the sequence of morphological changes

which causes gastric cancer is reasonably understood, molecular mechanisms underlying the intestinal metaplastic differentiation remain to be fully elucidated. Phenotypic changes that occur in IM appear to arise as a combination of modified expression of genetic factors, transcription factors (TF), signalling pathways, growth factors and epigenetic silencing (Gutierrez-Gonzalez and Wright, 2008).

Given that metaplasia involves the modification of a gastric to an intestinal phenotype, the study of digestive tract development is crucial to understand the mechanisms underlying this process. Indeed it is reasonable to hypothesize that some of them will be recapitulated in the pathological setting, at least to some extent. Embryo regional specification involves the regulation, by multiple TFs, of genes necessary for various specific developmental pathways. Understanding the function of these genes might help understand how the endoderm gives rise to different phenotypes along the GI tract, and provide clues on how IM develops.

In this review, the embryonic development of the GI tract will be addressed, with special emphasis on anterior-posterior (AP) patterning and on molecular pathways playing a role in its regulation. In the end, we will try to establish a parallel with the pathways that may be involved in the development of IM.

Embryonic development

The development of the GI tract follows a well-established sequence of steps, through which multiple signals mainly derived from the mesoderm pattern future intestinal domains according to four axes: anterior-posterior, dorsoventral, left-right and radial (Wells and Melton, 2000; McLin et al., 2009; Spence et al., 2011a,b). In most vertebrates, including humans, the primary germ layers originate from a primitive pluripotent layer of epithelial cells called epiblast. This in turn originates from the inner cell mass (ICM) and does not exhibit AP axis. The ICM also differentiates into a layer of extra-embryonic cells called the primitive endoderm, which surrounds the epiblast and gives rise to the visceral and parietal endoderm (Fig. 1). A group of cells from the visceral part, moving to the anterior segment of the embryo, forms the anterior visceral endoderm (AVE), defining the initial AP axis of the embryo (Zorn and Wells, 2007). During the third week of pregnancy gastrulation begins, leading to the formation of the three primary germ layers: endoderm, mesoderm and ectoderm (Schoenwolf and Larsen, 2009; Heath, 2010; Sadler et al., 2014). This process, which begins with the formation of the primitive streak from the posterior epiblast, originates the definitive endoderm, through gradual replacement of visceral endoderm cells (Lewis and Tam, 2006; Zorn and Wells, 2007).

Several factors are involved in the regulation of this trilaminate differentiation process. Nodal protein, a member of the TGF-β superfamily, plays a key role in this process. Nodal signalling is necessary and sufficient to initiate the development of the endoderm and mesoderm. Much evidence suggests that endoderm and mesoderm arise from a common precursor cell, the mesoendodermic cell (Lewis and Tam, 2006; Grapin-Botton and Constam, 2007; Zorn and Wells, 2007, 2009; Spence et al., 2011a,b). High Nodal exposure of mesoendodermic cells during gastrulation promotes endodermal differentiation, whereas low levels promote mesodermal differentiation (Vincent et al., 2003; Lewis and Tam, 2006; Grapin-Botton and Constam, 2007; Zorn and Wells, 2009). Wnt signalling seems to cooperate with Nodal at mesoendodermic standardization (Vincent et al., 2003; Grapin-Botton and Constam, 2007; Zorn and Wells, 2007). In mice, null mutants for β-catenin or Wnt3 do not suffer gastrulation (Marikawa, 2006). Furthermore, another member of the TGF-β superfamily, Activin, is able to mimic Nodal function by binding to the same receptors, promoting the formation of the definitive endoderm (Zorn and Wells, 2009; Cao et al., 2011).

After all primary germ layers become established, the endoderm undergoes a complex series of transformations that gives rise to the digestive tract, gradually standardized and differentiated into the various specific organs. Different signalling pathways that might

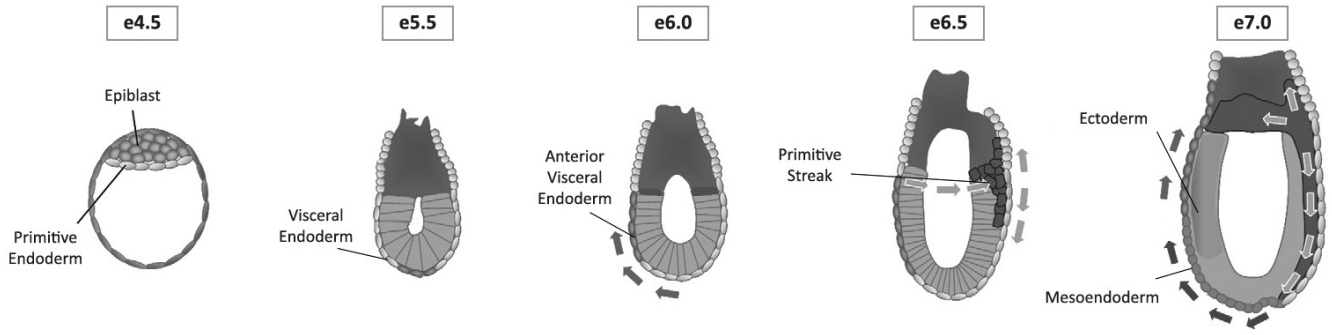

Fig. 1. Morphological overview of mouse early development. Thin arrows point the main structures in GI tract development. Thick arrows indicate key cell movements during formation of the three primary germ layers. See text for details. Adapted, with permission, from Yusuke Marikawa (2006).

regulate this regionalization process have been identified. In addition, several genes with temporally and spatially limited expression patterns were also identified (Kimura et al., 2011).

Through formation of two opposite endodermal invaginations, anterior intestinal portal (AIP) and posterior intestinal portal (PIP), and its junction at the midline, while the lateral endoderm folds ventrally, a primitive gut regionalized at a AP axis is formed - foregut cranially, hindgut caudally and midgut between them - by embryonic day 9 (e9.0) (mice) (Grapin-Botton and Melton, 2000; Lewis and Tam, 2006; van den Brink, 2007; Noah et al., 2011; Spence et al., 2011a,b). The outermost layer of this primitive tube is the ectoderm, the innermost layer is the endoderm, with the mesoderm between them. The endodermal layer will give rise to the intestinal epithelium. The mesoderm will condense and differentiate into lamina propria, submucosa, muscle tissue, vessels and connective tissue (Rubin, 2007; McLin et al., 2009; Schoenwolf and Larsen, 2009).

After the intestinal tract is formed, the simple epithelium condenses and converts into an undifferentiated pseudostratified epithelium (e9.5) (Spence et al., 2011a,b). The morphology of endodermal cells remains undifferentiated until e12 (mice). After morphogenesis, and continuing during the postnatal period, the multiple cell types of the GI tract originate from the undifferentiated endoderm. These cells generate the characteristic epithelium of each organ (Kim et al., 2005; McLin et al., 2009). The adult intestinal epithelium is then composed of two predominant cell groups, derived from multipotent stem cells: absorptive cells, designated enterocytes in the small bowel and colonocytes in the large bowel, and various types of secretory cells: goblet, tuft, Paneth, and enteroendocrine cells (Spence et al., 2011a,b). The cytodifferentiation phase coincides with the formation of the fifth axis of the GI tract, the villous-crypt axis, which is the functional bowel axis. This phase is characterized by the development of multiple invaginations into the lumen, the villi, comprised of a mesodermal base and an endodermal layer containing, among others, precursor cells of the different cellular lineages (McLin et al., 2009; Schoenwolf and Larsen, 2009; Yeung et al., 2011). Mesenchyme encompasses, among others, subepithelial myofibroblasts believed to be the major regulators of mesenchymal signalling (McLin et al., 2009; Yeung et al., 2011). Simultaneously with the elongation of the digestive tract, the intestinal epithelium undergoes an extensive restructuring process, at the rostro-caudal direction, to finally become a simple columnar epithelium overlaying the luminal surface of the bowel (Sancho et al., 2004; van den Brink, 2007; Noah et al., 2011; Spence et al., 2011a,b).

Following a series of rotational events that result in the correct positioning of the digestive tube within the abdominal cavity (Schoenwolf and Larsen, 2009; Sadler et al., 2014), the abdominal portion of the foregut originates the esophagus, stomach, and proximal duodenum (proximally to the ampulla of Vater). The midgut differs in the distal duodenum (distally to the ampulla of Vater), jejunum, ileum, cecum, ascending colon and proximal two-thirds of the transverse colon. The hindgut originates the distal third of the transverse colon, descending and sigmoid colon and rectum (McLin et al., 2009; Schoenwolf and Larsen, 2009).

Anterior-posterior patterning of the digestive tract

The patterning process is orchestrated by several families of TFs and can be divided into two stages, the first occurring during or even before gastrulation, and the second one after formation of the definitive endoderm (Heath, 2010).

The endoderm receives patterning signals even before gut morphogenesis (Wells and Melton, 2000; Dessimoz et al., 2006). In fact, there is AP asymmetry as early as e5.5-e6 at visceral endoderm (Zorn and Wells, 2007). Additionally, regional differences regarding expression of molecular markers can be detected at gastrulation stage (e7.5) (Moore-Scott et al., 2007; Grapin-Botton, 2005; Lewis and Tam, 2006; Zorn and Wells, 2009). These observations suggest that, at this stage, the endoderm has already been subdivided into separate regions that will result in the foregut, midgut and hindgut. Thus, despite the first AP polarity signal being the formation of the primitive streak and AVE at the beginning of gastrulation, specification of an AP axis appears to initiate even before gastrulation. Furthermore, expression of these markers persists during establishment of the digestive tract (Moore-Scott et al., 2007), suggesting that their early expression allows prediction of expression domains at a later stage. Accordingly, the position of endodermal progenitor cells in the primitive streak reflects their future anterior-posterior position (Grapin-Botton and Constam, 2007).

One of the key proteins involved at an early stage in AP patterning is Nodal. Its inactivation causes decreased expression of the *homeobox* gene *Hhex*, an anterior endodermal marker, and Shh, an anterior meso-endodermal marker (Vincent et al., 2003; Lu and Robertson, 2004). At later stages of gastrulation, cells in the posterior primitive streak are under the influence of lower levels of Nodal and tend to contribute to posterior endoderm (Lewis and Tam, 2006; Zorn and Wells, 2009). Furthermore, through an exposure gradient, Nodal also regulates expression of multiple TFs, including, among others, Foxa2 and "mix-like" (Mixl1) proteins (Zorn and Wells, 2007, 2009; Heath, 2010; Noah et al. 2011) which appear to be necessary to specify different regions of the endoderm: *Foxa2*$^{-/-}$ embryos exhibit defects in the foregut and midgut, while embryos lacking Mixl1 show defects in the hindgut (Grapin-Botton, 2005; Grapin-Botton and Constam, 2007; Zorn and Wells, 2007). Thus, besides its role during mesoendodermic differentiation, Nodal signalling appears to play a secondary role in anterior specification, granting anterior regional identity to the recently formed

endoderm.

After gastrulation, however, naive endodermal cells retain plasticity and are not yet programmed to differentiate into a specific tissue (Wells and Melton, 2000; Zorn and Wells, 2009; Sherwood et al., 2009). In fact, at this early stage of development, anterior endoderm cells (e7.5) cultured with posterior mesoderm express posterior markers and vice versa (Wells and Melton, 2000). Although not determined, endoderm is already patterned in different molecular domains, as shown by anterior expression of Hhex, Foxa2 and Sox2, and posterior expression of Cdx genes (Cdx1, Cdx2, and Cdx4), required for the development of the foregut and hindgut, respectively (Chawengsaksophak et al., 2004; Grapin-Botton, 2005; Sherwood et al., 2009; Noah et al., 2011). This gross regional differentiation is progressively refined to a point where, along somitogenesis, many TFs will define different territories that will differentiate in the esophagus, stomach, liver, duodenum, pancreas and small and large bowel (Grapin-Botton, 2000).

After formation of the primitive gut, the endoderm is additionally patterned by a set of (instructive and permissive) signals mainly from the adjacent mesoderm, which progressively subdivide it along the AP axis, first in foregut, midgut and hindgut, and finally in the primitive organs (Grapin-Botton, 2000, 2005; Sherwood et al., 2009). The gastric endoderm of the 4-day chicken embryo, when incubated with duodenal mesenchyme, differentiates into an intestinal-type epithelium, suggesting that mesenchyme is capable of inducing gastric epithelium differentiation into intestinal epithelium (Yasugi and Mizuno, 2008). However, the endoderm itself is also a source of patterning signals, proved by the implication of endodermal Sox2 in the establishment of pylorus-duodenal junction (Sherwood et al., 2009), as well as the boundary between the proximal squamous esophagus and stomach and the distal glandular stomach (Que et al., 2007). In fact, positional information is not only derived from the endoderm or mesoderm, but both seem to have partial information that changes throughout different stages of development.

In addition to Nodal, other pathways that appear to play a role in digestive tract AP patterning include Cdx, Wnt, Fgf, Bmp, Sox, retinoic acid (RA), Hedgehog, Hox, Barx1 and Runx3 (Fig. 2).

Cdx family

Mammalian embryos contain three *homeobox* genes related to the *Drosophila Caudal*, with overlapping expression patterns in the posterior region: *Cdx1, Cdx2* and *Cdx4* (Benahmed et al., 2008; Faas and Isaacs, 2009; Grainger et al., 2010). Due to this overlap there is probably a functional redundancy among *Cdx* genes and some degree of compensation can occur in deficiencies of its members (Faas and Isaacs, 2009). Both *Cdx1* and *Cdx2,* but not *Cdx4,* are also expressed in the epithelium of adult mouse and human intestine, from the duodenum to the rectum (Grainger et al., 2010; Kang et al., 2011) and play a role in epithelium development, maintenance, proliferation and AP patterning (Silberg et al., 2000; Guo et al., 2004).

The member of the ParaHox family *Cdx2* is perhaps the most important intrinsic TF in intestinal patterning and specification (Gao et al., 2009; Grainger et al., 2010; Silberg et al., 2000). In mice, its expression begins at e8.5 (Stringer et al., 2008; Grainger et al., 2010), predominantly in posterior endoderm. By e12.5 it is only expressed in the gut endoderm with a clear limit at the foregut-midgut junction (Silberg et al., 2000; Chawengsaksophak et al., 2004) and a more pronounced expression in the region that will originate the proximal colon, decreasing both anteriorly and posteriorly (Silberg et al., 2000; Chawengsaksophak et al., 2004; Guo et al., 2004) (Fig. 2). In contrast, its associated ParaHox gene *Cdx1* is only expressed in embryos from e12.5, with

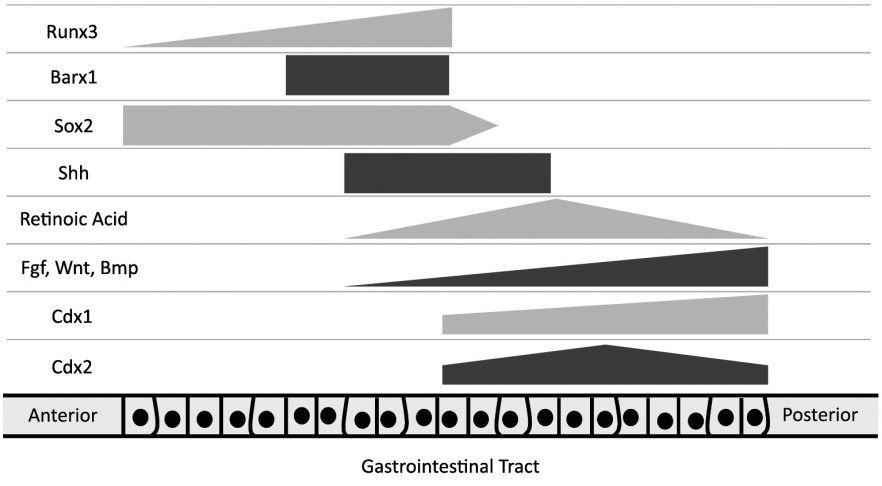

Fig. 2. Localization of several genes with a role on anterior-posterior patterning during normal development of gastrointestinal tract. It is noted an overlapping expression of various genes, both at anterior and at posterior later. Nonetheless, an anterior-posterior gradient of expression is evident, with some genes restricted to the anterior gastrointestinal tract (Sox2, Barx1 and Runx3) and others restricted to the posterior end (Cdx1, Cdx2, Fgf, Wnt, Bmp and Retinoic Acid).

increasing expression along the AP axis, reaching highest intensity at the distal colon (Fig. 2), and it does not appear to have a preponderant role in intestinal development (Silberg et al., 2000; Guo et al., 2004; Benahmed et al., 2008; Grainger et al., 2010). In contrast to previous evidence (Gao et al., 2009) it was shown that *Cdx1* expression is not affected by loss of Cdx2 by e13.5; however, *Cdx1 null* mutants showed a reduction of Cdx2 expression, consistent with a cross regulation between *Cdx* genes (Grainger et al., 2010).

In vivo knock-out (Chawengsaksophak et al., 2004) and overexpression studies (Mutoh et al., 2002) indicate that Cdx2 is involved in intestinal epithelium morphogenesis and differentiation, regulating Hox genes expression as well as intestinal differentiation genes (Almeida et al., 2003; Chawengsaksophak et al., 2004; Guo et al., 2004). Its deficiency seems to result in the formation of gastric mucosa rather than intestinal one (Stringer et al., 2008). Indeed, it was reported that expression of endodermal Sox2 and mesodermal Barx1, typically gastric genes (Kim et al., 2005, 2007; Tsukamoto et al., 2005; Que et al., 2007), was observed in the midgut of $Cdx2^{+/-}$ and $Cdx2^{-/-}$ embryos (Stringer et al., 2008). Expression of Barx1 in these embryos and the expression of intestinal genes, including Cdx2, in the gastric mucosa of Barx1 null mutants (Kim et al., 2007) indicate the existence of a negative feedback loop between Barx1 and Cdx2.

Cdx2 deletion in mice predominantly affects intestinal formation and growth. Gao et al. showed that gut lacking Cdx2 suffered a process of anterior transformation to a squamous type epithelium characteristic of the esophagus (Gao et al., 2009). This is consistent with the activation of a foregut differentiation program in the posterior region of the digestive tract and suggests that Cdx2 normally represses foregut differentiation in the posterior gut. Moreover, it was shown that Cdx2 is essential for early expression and/or subsequent maintenance of various pro-intestinal TFs, including Cdx1. In fact, although some controversy remains, Cdx1 is absent in the intestine of conditional Cdx2 knock-outs, reinforcing its dependence on Cdx2 expression and reflecting their sequential expression during endoderm development. Surprisingly, in the same study, it was found that in a Cdx2 deficiency-state, the primary enteric Hox code persisted and, in addition, the expression of other genes such as Barx1 remained unchanged (Gao et al., 2009). These findings support the idea that Cdx2 deficient bowel, despite suffering a posterior to anterior transformation, still maintains some AP identity. In another study, Grainger et al. showed that Cdx2 loss in mice at a later stage (e13.5) led to partial conversion of the intestinal epithelium into a pyloric identity with increased expression of gastric markers, including Sox2, and decrease of another intestinal marker, IFABP (Grainger et al., 2010). Taken together, these results support the hypothesis that Cdx genes, especially Cdx2, are essential in the establishment of an intestinal identity (Fig. 3) and that loss of Cdx2 affects the intestinal patterning on a time-dependent manner, with early loss resulting in a more marked anterior transformation.

Cdx2 appears initially necessary to establish an endodermal posterior identity and to suppress an anterior identity and, at a later stage, to maintain the AP axis (Gao et al., 2009; Grainger et al., 2010). This suggests that a combination of molecules responsible for the AP patterning, described ahead and including Wnt, Fgf, Bmp and RA, incorporate their signalling and regulate Cdx2 activity on the endoderm, with Cdx2 acting as a point of convergence of these signalling pathways (Fig. 3). Mechanisms whereby Cdx2 expression is regulated are complex and still widely unknown. Furthermore, different signalling pathways probably have different effects on Cdx2 expression at various developmental stages.

Fgf family

Fgf4 appears to have an important role in the anterior-posterior patterning of the gut, both during gastrulation and at later stages (Wells and Melton, 2000; Dessimoz et al., 2006). Fgf signalling is required for normal expression of Cdx genes in the gastrula stage (Keenan et al., 2006). In mice and chicken it was demonstrated that an endoderm that does not receive Fgf4 signals develops foregut characteristics. On the other hand, posterior endoderm is progressively programmed upon exposure to higher concentrations of this TF, through Cdx expression (Fig. 3) and inhibition of expression of anterior genes such as Hhex and Foxa2. Indeed, endoderm exposure in the late gastrula stage to Fgf4 results in the anterior expression of genes that are

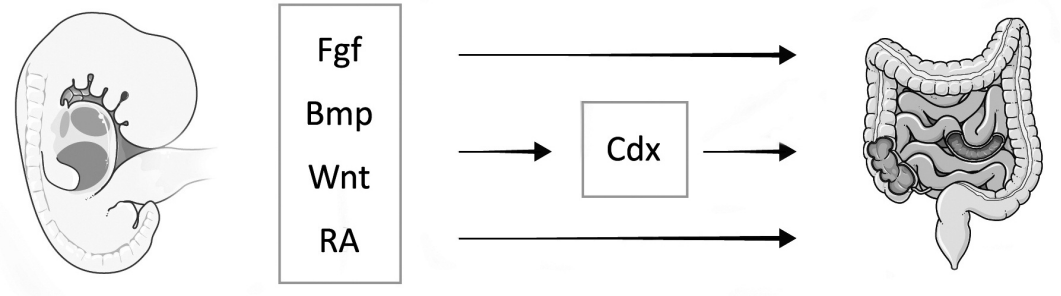

Fig. 3. Transcription factors involved in intestinal differentiation. Note that the multiple signaling pathways may, on one hand, directly regulate this process, or operate over Cdx, behaving this as a point of convergence of the different signaling pathways.

normally posterior, such as Cdx and Pdx1 (Wells and Melton, 2000; Dessimoz et al., 2006). It was also reported that Fgf2, and not Fgf4, may be the main Fgf signal with patterning functions of human stem cells (Ameri et al., 2010).

Bmp family

Bmps are signalling proteins belonging to the TGF-β superfamily that are crucial for normal intestinal development, contributing to its patterning, development and differentiation (De Santa Barbara et al., 2005; Barros et al., 2008). Experiments in zebrafish, Xenopus and chicken suggest that during the early stages of development Bmp signalling promotes development of posterior endoderm (Tiso et al., 2002; De Santa Barbara et al., 2005; Wills et al., 2008). In the gut, this signalling pathway, through Bmp2 and Bmp4, is a key mediator of the differentiation and architecture of intestinal epithelium. Furthermore, expression of Noggin, a Bmp pathway antagonist, in the mesenchyme ensures maintenance of the undifferentianted compartment.

Wnt family

Wnt signalling is evident early in gastrointestinal development and by e6.5 is already regulated in a differentiated manner in the visceral endoderm, stronger in the posterior side than in the anterior one (Marikawa, 2006). It was shown that Wnt signalling activates Cdx2 in the endoderm (Fig. 3), leading to a posterior migration of definitive endoderm by the induction of an intestinal genetic program in the anterior endodermal cells (Sherwood et al., 2011). Tcf1 and Tcf4 double mutants, at e8.5, do not form the PIP and lose expression of posterior endoderm markers (Gregorieff et al., 2004). In zebrafish and frogs, Wnt/β-catenin, similarly to Fgf, is necessary and sufficient to promote posterior intestinal development and inhibit anterior development (Zorn and Wells, 2009).

Stem cell-derived endoderm exposed to Wnt3a increases the expression of Cdx2 (Cao et al., 2011). Also, it was shown that expression of Wnt3a and Wnt5a decreases in response to Cdx decrease, and that increasing Cdx expression increases the expression of Wnt genes (Faas and Isaacs, 2009). Together with the fact that mice lacking expression of Wnt3a or of Cdx2 exhibit similar phenotypes (Chawengsaksophak et al., 2004), we can infer the existence of a positive feedback loop between these two pathways for posterior development. Although it was shown that Wnt activation is sufficient to induce intestinal differentiation (Sherwood et al., 2011), this is in disagreement with another study where simultaneous expression of Wnt and Fgf signals was required for the induction of intestinal differentiation (Spence et al., 2011a,b).

At this point, we can infer that differentiation of anterior structures, such as the stomach, requires suppression of the Wnt pathway (Kim et al., 2005, 2007) while posterior gut development is dependent on Wnt (Gregorieff et al., 2004).

Sox family

Sox2 is normally expressed in the gastric mucosa and is absent from the intestinal epithelium (Tsukamoto et al., 2005; Que et al., 2007; Camilo et al., 2015). Its expression is reduced in IM, namely of the complete type, and in more advanced lesions in the carcinogenesis cascade such as gastric dysplasia (Tsukamoto et al., 2005; Camilo et al., 2015). Also, upon *H. pylori* infection, a presumed triggering factor of IM, gastric mucosa of mice exhibits upregulation of an intestinal differentiation program and downregulation of a gastric one, with decreased expression of Sox2, which may eventually lead to the onset of IM (Camilo et al., 2012).

Other signalling pathways

Retinoic acid promotes the formation of hindgut (Duester, 2008) directly stimulating the expression of Cdx genes (Bayha et al., 2009) (Fig. 3). On the other hand, the foregut only forms in the absence of RA (Bayha et al., 2009).

Mutations in Hox genes indicate that they are required for GI tract development: anterior expression of a normally posterior gene, Hoxa13, results in the transformation of the gastric epithelium into an intestinal one (Grapin-Botton, 2005) and abnormal expression of Hoxd13 in the chicken midgut causes hindgut development (McLin et al., 2009).

Recent studies demonstrate the possible existence of additional regulators of Cdx2 expression, namely Pinin (Joo et al., 2010), ISX (Sue et al., 2016) and NKX6.3 (Yoon et al., 2016).

Transcription factors involved in intestinal metaplasia development

Both Cdx1 and Cdx2 are restrictively expressed in small bowel and colon epithelium and are also ectopically expressed in gastric IM (Silberg et al., 2002; Almeida et al., 2003). Ectopic expression of Cdx2 in mouse stomach results in transformation of the gastric mucosa into an intestinal-type mucosa, which is evidenced by the presence of typical intestinal cells and genes, similar to what happens in human IM. This evidence shows the sufficiency of Cdx2 *homeobox* gene in ectopic intestinal epithelial specification and its central role in the induction of IM (Mutoh et al., 2002; Silberg et al., 2002). Similarly to Cdx2, it was shown that transgenic mice with gastric Cdx1 expression also develop IM (Mutoh et al., 2004). The association of Cdx1 and Cdx2 with MUC2 expression (Almeida et al., 2003), a marker of intestinal differentiation (Reis et al., 1999), and its increased expression in gastric dysplastic and neoplastic tissues (Kang et al., 2011), are additional facts supporting the hypothesis that these genes are

involved in gastric carcinogenesis. Taken together, these results suggest that Cdx genes have a fundamental role, through independent programs, in the trans-differentiation of the gastric mucosa to an intestinal type characteristic of IM. The maintenance of intestinal differentiation appears also dependent on the presence of Cdx2.

Based on above mentioned results, it seems reasonable to suggest that the gradient of Fgf mesodermal expression that starts during gastrulation may be involved in the establishment of Cdx2 expression domains, resulting in final specification of the intestinal mucosa. Accordingly, its deregulation could be involved in the transdifferentiation metaplastic process.

BMP2 and BMP4 TFs are increased and active along with Cdx2 in IM and upregulate its expression (Fig. 3), as well as that of MUC2, through signalling transducers such as Smad4 (Barros et al., 2008). These data may also indicate a significant role of Bmp family in IM origin.

Regarding the Wnt pathway, at later stages of development repression of Wnt signalling results in incomplete intestinal development with reduced Cdx2 expression and a posterior expansion of the anterior marker Sox2, consistent with an anterior displacement of the GI tract (Gregorieff et al., 2004), a phenotype very similar to the one obtained with loss of Cdx2 at e13.5 (Grainger et al., 2010). On the other hand, anterior Wnt activation can cause IM, similar to that observed in humans (Grapin-Botton, 2005).

Gastric TFs such as Sox2 may also be involved in IM development. Very recently, it was shown that Sox2 KO stomach exhibits upregulation of a subset of genes associated with intestinal homeostasis and metaplasia and downregulation of gastric ones (Sarkar et al., 2016). These data may indicate that a mutual inhibition between Sox2 and Cdx2 may have a relevant role in lower digestive tract homeostasis as well as in the origin of IM.

Also in stomach, the expression of Shh, a member of the Hedgehog family, is reduced by *H. pylori* infection and is absent in IM (van den Brink, 2007). Moreover, $Shh^{-/-}$ mice exhibit intestinal transformation of the stomach (Ramalho-Santos et al., 2000) which together indicate that Shh might be involved in IM development. However, in mutant mice, goblet cells were not observed in the epithelium nor was expression of either Cdx2 or Muc2 documented, while gastric markers remained expressed. Altogether, available data suggest that Shh may be important to restrict the expression of gastric markers but may not suffice to induce IM.

Gastric specification needs Barx1, a TF exclusive of the gastric mesoderm. Barx1 loss of function profoundly affects gastric differentiation, with ectopic expression of intestinal markers (Cdx2) in the distal stomach (Kim et al., 2005, 2007). Barx1 expression is transient, occurring only during organogenesis, thus it is unlikely to participate in the maintenance of the gastric mucosa or prevention of IM in adults. However, IM may partly result from the injury induced by reactivation of the Wnt signalling in adult life when Barx1 is not present to inhibit it (Kim et al., 2005).

In humans, Runx3 expression is markedly decreased in IM (Li et al., 2002), and in mice, $Runx3^{-/-}$ gastric epithelial cells differentiate *in vivo* into intestinal-type cells with Cdx2 expression. This puts forward the hypothesis that the loss of Runx3 may be a triggering event for gastric Cdx2 expression with resultant intestinal differentiation, and that in normal gastric mucosa Cdx2 is inhibited, directly or indirectly, by Runx3 (Fukamachi et al., 2004).

Cross-regulation between different signalling pathways

The available information supports a model in which proteins such as Cdx, Wnt, Bmp and Fgf4 progressively establish posterior regions at the endoderm. The way these multiple signalling pathways interact with each other and in particular in Cdx2 regulation, both in normal intestinal mucosa and in metaplastic lesions, is largely unclear. However, several molecular interactions are already evident.

Besides Cdx2 regulation by several pathways, the existence of cross-regulation between other TFs has been suggested. It was shown that synergistic activity between Wnt and Fgf is necessary to induce sustained formation of posterior endoderm lineage with Cdx2 expression (Keenan et al., 2006; Spence et al., 2011). Fgf4 and RA also appear to cooperate synergistically in endoderm posterior induction, stimulating Cdx2 expression (Johannesson et al., 2009; Camilo et al., 2012). Other endodermal TFs, namely from the Sox family (Sox2 and Sox9), counterbalance the effect of these posteriorizing TFs, suggesting that the demarcation between gastric and intestinal mucosa may involve the balance between them (Blache et al., 2004; Benahmed et al., 2008). Indeed, it seems clear that an antagonism between an intestinal promoter pathway Wnt/Cdx2 and Barx1/Sox2 governs the establishment of a demarcation line between stomach and gut (Stringer et al., 2008; Sherwood et al., 2009).

Another possible mechanism through which various TFs, including Cdx2, control the identity of diverse segments along the GI tract is by direct regulation of Hox gene expression in specific areas of the AP axis (Grapin-Botton, 2005). Hox genes may possibly integrate all the regulatory information and provide positional information along the mouth-anus axis.

Conclusions

The study of intestinal development, particularly at the molecular level, is still an area of limited knowledge but with a great potential to allow us to understand intestinal metaplasia development and, from there, enable us to intervene in its progression.

Cdx genes, namely Cdx2, appear to be the main regulators of normal intestinal differentiation and also to

be largely involved in the metaplastic epithelial transdifferentiation process. However, control of gene expression both along intestinal development and in IM is complex and seems to depend on several families of signalling molecules besides Cdx, namely Fgf, Bmp and Wnt. In fact, genes involved in normal intestinal differentiation are unquestionably involved in IM development. As knowledge in this area increases, the number of potential players in this process enlarges and, on the other hand, contradictory results persist as obstacles for the clear definition of the mechanisms involved in IM.

Considering that IM is a major precursor lesion of gastric dysplasia and cancer, further studies are needed to clarify the mechanisms underlying IM, with the aim of preventing neoplastic development.

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