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Antero-posterior patterning of the vertebrate digestive tract: 40 years after Nicole Le Douarin's PhD thesis

ANNE GRAPIN-BOTTON*

Swiss Institute for Experimental Cancer Research (ISREC), Epalinges, Lausanne, Switzerland

ABSTRACT This review is dedicated to the work on chick digestive tract organogenesis that Nicole Le Douarin performed as a PhD student under the direction of Etienne Wolf. I discuss how she laid the grounds for future work by establishing fate maps at somitic stages, by describing morphogenetic movements between germ layers and by pointing to signaling events between endoderm and mesoderm. Her inspiring work was extended by others, in particular at the molecular level, leading to a better understanding of antero-posterior patterning in the digestive tract. Antero-posterior patterning of endoderm is initiated at gastrulation when future anterior and posterior endoderm ingress at different times and accordingly express different genes. Plasticity is however maintained at somite stages and even later, when organ primordia can be delineated. There is a cross-talk between endoderm and mesoderm and the two layers exchange instructive signals that induce specific antero-posterior identities as well as permissive signals required for organogenesis from previously patterned fields. Recent experiments suggest that several signaling molecules involved in neural tube antero-posterior patterning are also instrumental in the digestive tract including retinoic acid and FGF4.

KEY WORDS: *endoderm, gut, axis, signaling, chick*

Introduction

Nicole Le Douarin's name is often associated with quail-chick chimeras and the fantastic jump triggered by the use of this technique to study neural crest cell derivatives. Less is known about her early work on digestive tract organogenesis, which started when she was a PhD student under the direction of Etienne Wolf in Nogent. The fact that this work was mainly published in French makes it less accessible but nevertheless very insightful. The purpose of this review is to draw attention to her early work and show how it inspired developmental biologists since then. The first of her main achievements was the establishment of a fate map of the 10-25-somite-stage chick endodermal and mesodermal territories that give rise to different organs of the digestive tract and the observation of interesting sliding movements between these two layers. The second contribution concerns the requirement for interactions between endoderm and mesoderm during the specification of domains in the digestive tract. This work was pursued in many labs including those of Michèle Kedinger, Kathy Haffen, Sadao Yasugi and more recently ours. In particular, N. Le Douarin demonstrated the requirements for cardiac mesoderm and later septum transversum in liver induction and organogenesis. These findings were recently extended to the mouse and studied at the molecular level by Ken Zaret and coworkers. Forty years later, our overall comprehension of how organs are laid out along the antero-posterior (A-P) axis is less extensive in the digestive tract than in the neural tube with which parallels in the mechanism and molecules involved can nevertheless already be drawn.

A crude and unstable A-P asymmetry in endoderm after gastrulation

How organ formation correlates with specific regions of the flat endodermal sheet has been a recurrent question, asked as early as 1874 when His published a map of the presumptive digestive and respiratory organs of the chick blastoderm (His, 1874). Fate-mapping experiments have traced the fate of cells in the definitive digestive organs prior to or just after gastrulation in many species.

Abbreviations used in this paper: AIP, anterior intestinal portal; AP, antero-posterior; CIP, caudal intestinal portal; IFABP, intestinal fatty acid binding protein; LPM, lateral plate mesoderm.

***Address correspondence to:** Dr. Anne Grapin-Botton. Swiss Institute for Experimental Cancer research (ISREC), Chemin des Boveresses 155, Case Postale CH-1066 Epalinges s/Lausanne, Switzerland. Fax: +41-21-652-6933. e-mail: anne.grapin@isrec.ch

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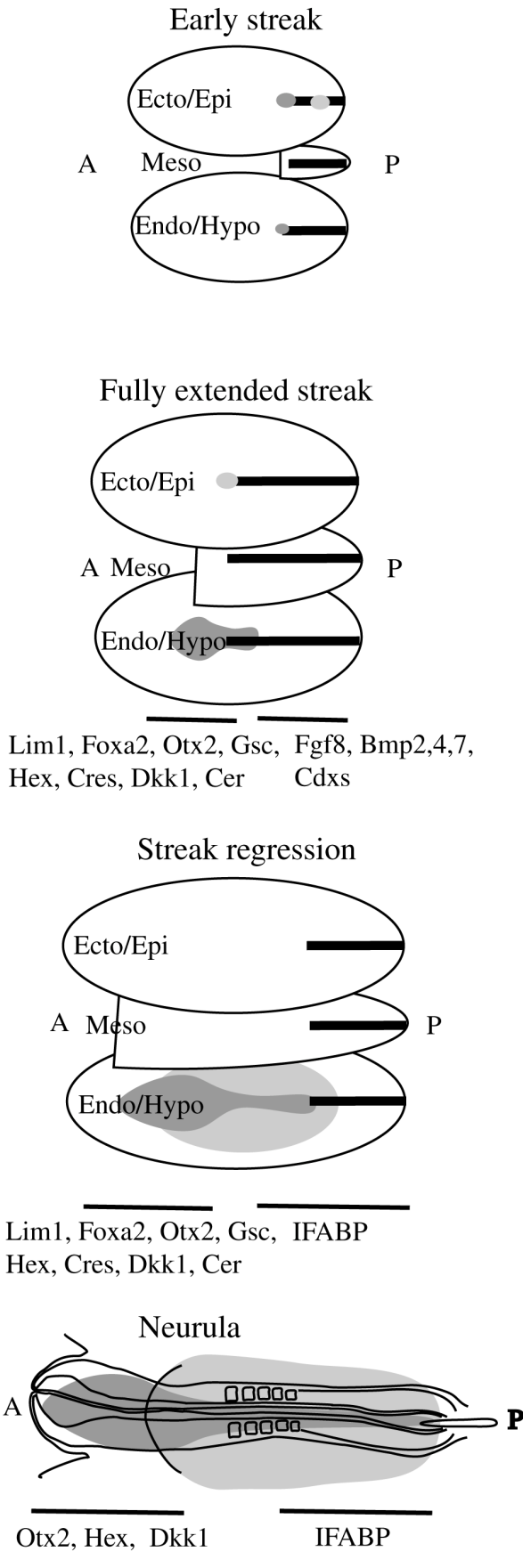

As early as the 32-blastomere stage in *Xenopus* it was shown that ventral vegetal blastomeres tend to give progeny in more posterior endoderm areas than dorsal blastomeres (Dale and Slack, 1987; Moody, 1987) In zebrafish, labeling of single cells located in the margin of the 1000/3000-cell gastrula reveals that different regions give rise to a specific subset of organs but single cells contribute to several organs (Warga and Nusslein-Volhard, 1999). In chick, fate-mapping of endoderm precursors in epiblast and primitive streak have shown that at early streak stage endoderm precursors are found throughout the streak and become restricted to the tip of the streak by late streak stage (Hatada and Stern, 1994, Psychoyos and Stern, 1996)(and abundant former literature herein)(Lawson and Schoenwolf, 2003; Kirby *et al.,* 2003). The most posterior streak endoderm precursors give rise to more posterior and lateral endoderm (Lawson and Schoenwolf, 2003, Rosenquist, 1972). These results are compiled in figure 1. In the mouse, Lawson and co-workers traced the derivatives of the epiblast (Lawson *et al.*, 1986, Lawson *et al.*, 1991) and outer layer (Lawson and Pedersen, 1987) in prestreak and early streak stage embryos using horseradish peroxydase injections (6.7 and 7.5 days *post-coitum*, dpc). As in chick, definitive endoderm cells derive from the distal PS and gradually replace the primitive endoderm, starting with the most anterior cells (Kinder *et al.*, 2001). Fate mapping and gene expression analysis also indicate that different endoderm populations exit the streak in a defined order, starting with *Hex(Hematopoietically expressed homeobox)* -positive cells which colonize mainly the ventral foregut (Thomas *et al.*, 1998) and followed by cells expressing the forkhead transcription factor *FoxA2*, forming dorsal foregut, midgut and eventually the hindgut (Ang and Rossant, 1994, Dufort *et al.*, 1998, Lawson *et al.*, 1986).

Many other markers are asymetrically expressed after gastrulation as shown in Fig. 1. As groups of mesodermal and endodermal cells remain associated during and after gastrulation, gene expression is often reported in mesendoderm rather than endoderm (Parameswaran and Tam, 1995) (Kinder *et al.*, 1999, Tam *et al.*, 1997). Functionally, A-P asymmetry is demonstrated

Fig. 1. Spatial organization of endoderm at gastrulation and headfold stage in the chick. *The three layers are schematically dissociated, epiblast/ectoderm on top, mesoderm in the middle, hypoblast/endoderm at the bottom. Anterior/midline endoderm is in dark grey and posterior/ lateral endoderm is in pale grey. The presence of endodermal cells in transit in the middle layer has not been assessed. The dark line is the primitive streak. A refers to anterior and P to posterior. Anterior markers are the transcripts for the secreted proteins* Cerberus 1 (Cer) *(Belo* et al.*, 1997; Biben* et al.*, 1998; Chapman* et al.*, 2002),* Dickkopf1 *(Foley* et al.*, 1997; Glinka* et al.*, 1998; Pearce* et al.*, 1999),* Crescent *(Pfeffer* et al.*, 1997) and the homeobox transcription factors* Orthodenticle homeobox (Otx) *2 (Ang* et al.*, 1994; Bally-Cuif* et al.*, 1995),* Goosecoid *(Izpisua-Belmonte* et al.*, 1993; Blum* et al.*, 1992),* Hex *(Thomas* et al.*, 1998; Yatskievych* et al.*, 1999) and* Lim Homeobox 1 (Lim1) *(Chapman* et al.*, 2002; Shawlot and Behringer, 1995) as well as* FoxA2 *(Alexander and Stainier, 1999; Ang* et al.*, 1993; Ruiz i Altaba* et al.*, 1995),* Her5 *(Bally-Cuif* et al.*, 2000). Many of these genes are also expressed in the primitive endoderm in the AVE in addition to definitive endoderm (Rodriguez* et al.*, 2001). Posterior markers of endoderm are more scarce but one of them is* Intestinal fatty acid binding protein *transcript* (IFABP) *(Wells and Melton, 2000). Many posterior markers like* Fgf8, Bmp 2,4,7, Cdxs *(Chapman* et al.*, 2002; Marom* et al.*, 1997) are expressed in the posterior streak where layers can not be distinguished.*

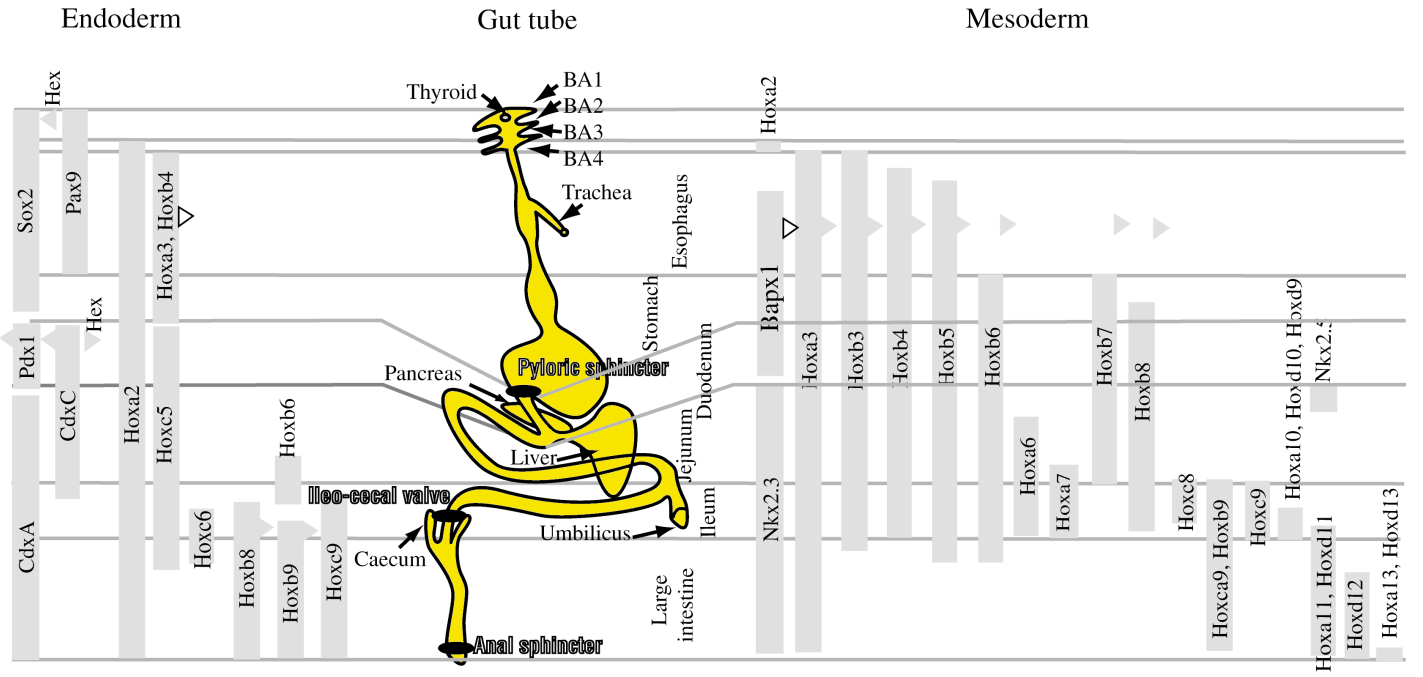

Fig. 2. Regionalization of endoderm from somitogenesis to organogenesis. *Many transcription factors are expressed in restricted domains of endoderm and mesoderm. This pattern progressively arises as somitogenesis begins. The gene expression boundaries are based on in situ hybridization on sections performed by the author and published data, all at embryonic day 4 (E4) in the chick (Grapin-Botton and Melton, 2000, Sakiyama* et al.*, 2000, Sakiyama* et al.*, 2001, Yokouchi* et al.*, 1995). There are some discrepancies with other sources using PCR for detection, different stages and species (Beck* et al.*, 2000). The triangles represent the thyroid (left), trachea and lungs (right), liver (right), pancreas (left) and caeca from up to down.*

by the specific ability of the anterior endoderm to induce heart differentiation in the mesoderm at the same stages (Marvin *et al.*, 2001, Narita *et al.*, 1997, Schultheiss *et al.*, 1995). The initial formation of the rostral and caudal endoderm is affected by different genes. When the forkhead transcription factor *Sox17* or the BMP signaling pathway genes *FoxH1* or *smad2* are inactivated, only the posterior gut, ingressing later, is affected (Hoodless *et al.*, 2001, Kanai-Azuma *et al.*, 2002, Tremblay *et al.*, 2000, Yamamoto *et al.*, 2001). On the contrary, *FoxA2* -knockout cells can form hindgut but not fore- and midgut (Dufort *et al.*, 1998).

The segregation between anterior and posterior precursors also takes place very early in several invertebrates. In sea urchin, two types of endoderm precursors are segregated prior to gastrulation, veg1 and veg2 cells that contribute respectively to the archenteron base and tip (Logan and McClay, 1997) and accordingly gastrulate at different times. In *C. elegans*, the endodermal precursor, the E cell divides along the antero-posterior axis to give rise to Ea and Ep prior to gastrulation. Ea eventually gives rise to anterior gut derivatives and Ep to the posterior gut. This pattern is intrinsic to the cells since ablation of Ea or Ep ablates anterior or posterior gut respectively (Schroeder and McGhee, 1998). Anterior and posterior endoderm anlage are also segregated early in *Drosophila* (Reuter *et al.*, 1993).

In spite of the regional expression of genes, their expression is still plastic.Wells and Melton (2000) showed that at 7.5dpc anterior endoderm associated with posterior mesendoderm turns on several posterior markers and off some anterior ones (Wells and Melton, 2000). The converse is also true. Posterior specification might be mediated in part by fibroblast growth factor (FGF) 4, a secreted protein expressed in the posterior half of the embryo at this time (Niswander and Martin, 1992, Wells and Melton, 2000). *Fgf4* inactivation is deleterious shortly after implantation, so far precluding a validation for its requirement in A-P patterning in vivo (Feldman *et al.*, 1995).

As somites form, a complex gene expression map prefigures organogenesis in endoderm and mesoderm

Some of the early anterior endoderm markers are lost between gastrulation and neurulation as it is the case for *Gsc*, *Lim1* and *Crescent*, Others like *Hex* are maintained. A last category is turned on as somitogenesis begins, like *Pdx1*, *Cdx2/A*, *Sox2* and *3*, *Nkx2.1* and *Hox* genes. According to fate mapping experiments in the chick and to their maintenance during organogenesis, it can be inferred that they are restricted to the precursor fields of specific organs. In the 15-somite-stage chick, domains that contribute to various digestive organs do overlap although regions of different fates can be distinguished (Le Douarin, 1964b, Matsushita, 1996, Matsushita, 1999). There is only one fate mapping study at this stage in the mouse and it largely corroborates the chick data (Tremblay and Zaret, 2005).

Accordingly there are at least 6 domains with different gene expression profiles at this stage and they are maintained after organogenesis (Fig. 3) (Grapin-Botton and Melton, 2000).

As *Hox* genes play a major role in A-P patterning in other germ layers, a particular attention should be given to these genes. Most of the Vertebrate *Hox* genes are expressed in splanchnic mesoderm but only a subset is expressed in the endoderm at

levels detectable by *in situ* hybridization (Fig. 2). Similarly, in *Drosophila*, *labial* is the only *Hox* gene expressed in the endoderm whereas more *Hox* genes are expressed in mesoderm. The anterior expression limits of *Hox* genes in the endoderm do not always correlate with boundaries between organs but there are hotspots of *Hox* boundaries at the levels of sphincters (pyloric, ileo-caecal, anal) (Roberts, 2000). *Hox* gene inactivation may lead to digestive tract malformations as reported for *Hoxa3* (Manley and Capecchi, 1995, Manley and Capecchi, 1998), *Hoxa5* (Aubin *et al.*, 1999, Aubin *et al.*, 2002, Aubin *et al.*, 1997), *Hoxc4* (Boulet and Capecchi, 1996), *Hoxa13* and *Hoxd13* (Warot *et al.*, 1997) and *Hoxa4* (Tennyson *et al.*, 1993, Tennyson *et al.*, 1998). Their inactivation does generally not result in homeotic transformations, possibly due to redundancy although redundancy does not preclude from observing homeotic transformations of vertebrae in the mesoderm. Among experimental anterior expansions of *Hox* gene expression, only that of *Hoxa13* in the mesenchyme leads to induction of hingut fate in the midgut (Roberts *et al.*, 1998). Others induce malformations rather than posterior transformations (Pollock *et al.*, 1992, Tennyson *et al.*, 1993, Tennyson *et al.*, 1998). There is only one example of *Hox* gene playing a direct role in endoderm which concerns *Hoxa13* in the hindgut (de Santa Barbara and Roberts, 2002).

Another homeobox gene complex, the so-called *ParaHox* cluster, shown in *Amphioxus* to contain homologues for the *genomic screened homeobox (Gsh1)* gene, *pancreatic-duodenum-homeobox 1* (*Pdx1*) and *Caudal* homologue *Cdx2*, might play a role in A-P patterning (Brooke *et al.*, 1998). This cluster although not strictly endoderm-specific seems to be mostly implicated in endoderm development. Although *Gsh1* is not expressed in endoderm is Vertebrates, it is expressed in endoderm in other groups, including Cnidarians (Yanze *et al.*, 2001). The genes are expressed orderly along the A-P axis, *Gsh* being the most anterior and *Cdx2* the most posterior. Gain- and loss-of-function experiments demonstrated that in other germ layers than endoderm, *Cdx 1/ Xcad3* directly activates *Hox* genes and it may have the same properties in endoderm (Bel-Vialar *et al.*, 2002, Charite *et al.*, 1998, Epstein *et al.*, 1997, Isaacs *et al.*, 1998, Pownall *et al.*, 1996, van den Akker *et al.*, 2002). It may as well be a repressor of some *Hox* genes since the posterior expression boundaries of *Hoxa3* and *Hoxb4* correspond to the anterior limits of *Pdx1* and *CdxC/Cdx2* expression (Fig. 2). Gene inactivation of *Cdx2* clearly results in homeotic transformations. *Cdx2* heterozygotes exhibit induction of stomach tissue in the mid- and hindgut (Beck *et al.*, 1999). Stomach is the identity of the digestive tract immediately anterior to *Cdx2* expression domain. *Pdx1* inactivation results in pancreas atrophy and defects in the most anterior duodenum but it is not an anterior transformation (Offield *et al.*, 1996). Whether their effect is strictly relayed by *Hox* genes is unlikely since *Pdx1* and *Cdx2* directly activate genes playing a role in adult organ function (insulin (Ohneda *et al.*, 2000), lactase-phlorizin (Troelsen *et al.*, 1997), sucrase isomaltase (Suh *et al.*, 1994).

Morphogenetic events and their relation to A-P patterning

The gene expression patterns described above are established as complex morphogenetic movements shape the gut tube from a flat (as in chick or human) or cup-shaped (as in rodents) layer of cells. The mouse and chick gut tubes form from a crescent-shaped fold, the so-called anterior intestinal portal (AIP), which appears in the endoderm at the anterior tip of the embryo when somitogenesis begins (Fig. 3)(Bellairs, 1953, His, 1874). This fold progresses posteriorly. A similar fold, called the caudal intestinal portal (CIP), arises later at the posterior end of the embryo and moves anteriorly (Fig. 3)(Gaertner, 1949, Gasser, 1880). The two folds meet at the yolk stalk. N. Le Douarin used carbon particles to mark the AIP between 8 and 12 somite-stage and observed that a streak of particles was deposited in the ventral midline from esophagus to preumbilical small intestine, including the liver. This finding was recently corroborated in the mouse (Tremblay and Zaret, 2005). This suggest that the AIP is not only an area where lateral tissues meet as originally suggested by His (His, 1874) but functions as a zipper in which material from the AIP is deposited along the ventral midline as previously proposed by (Robinson, 1903) (Funccius, 1909) (Frazer, 1916) (Ludwig, 1919). Whether this material corresponds to cells endowed with high proliferation remains to be determined. If cells are deposited all along the ventral midline they will eventually colonize different axis levels although they originate from the same position. During AIP or CIP progression, they must acquire a pattern that is similar to that of the dorsal cells of the same level which derive from midline endoderm and have roughly maintained their position relative to the notochord and neural tube. In *Xenopus*, as the suprablastoporal lip of the blastopore forms the dorsal gut tube and the subblastoporal lip forms its ventral aspect, A-P identity also needs to be coordinated (Keller, 1975, Keller, 1976, Nieuwkoop, 1997). In Zebrafish, organ domains are segregated prior to gut tube formation (Wallace and Pack, 2003). The tube forms through the reorganization of cells that become polarized and form a lumen. This happens in the intestines first and much later in the pharynx and esophagus.

An interesting observation relative to the signals exchanged by endoderm and mesoderm (see below) is the fact that the endoderm slides posteriorly on the mesoderm (Fig. 3). For instance, N. Le Douarin showed that carbon particles put through the 3 germ layers at the level of somite 2 end up in the neck in the neural tube, at the level of the larynx in mesoderm and more posteriorly in brunchi in endoderm (Le Douarin, 1964; Tremblay and Zaret, 2005). This general posterior sliding of endoderm relative to mesoderm and mesoderm relative to neurectoderm is more pronounced posteriorly and laterally (Catala *et al.*, 1996, Le Douarin, 1964a). Accordingly, at the molecular level, anterior boundaries of *Hox* genes are more rostral in the neurectoderm, then somites, then lateral plate, then endoderm (Fig. 3) (Burke *et al.*, 1995). A shift in the same direction has been reported between ectoderm and visceral mesoderm in *Drosophila* (Tremml and Bienz, 1989).

In addition to these movements, Smith and Tabin (Smith and Tabin, 2000) showed that mesodermal cells migrate individually before 13-somite-stage and can thus clonally contribute to different gut organs. After this stage, although clones can populate different radial layers, they are restricted to one organ.

The mesoderm sends permissive signals to endoderm

Cultured alone, the endoderm survives very poorly (Le Douarin and Bussonnet, 1966, Okada, 1953, Okada, 1954a, Okada, 1954b, Sumiya and Mizuno, 1974, Takata, 1960). Of the few cases where culture of endoderm alone was performed it was either very short term (Kumar *et al.*, 2003, Wells and Melton,

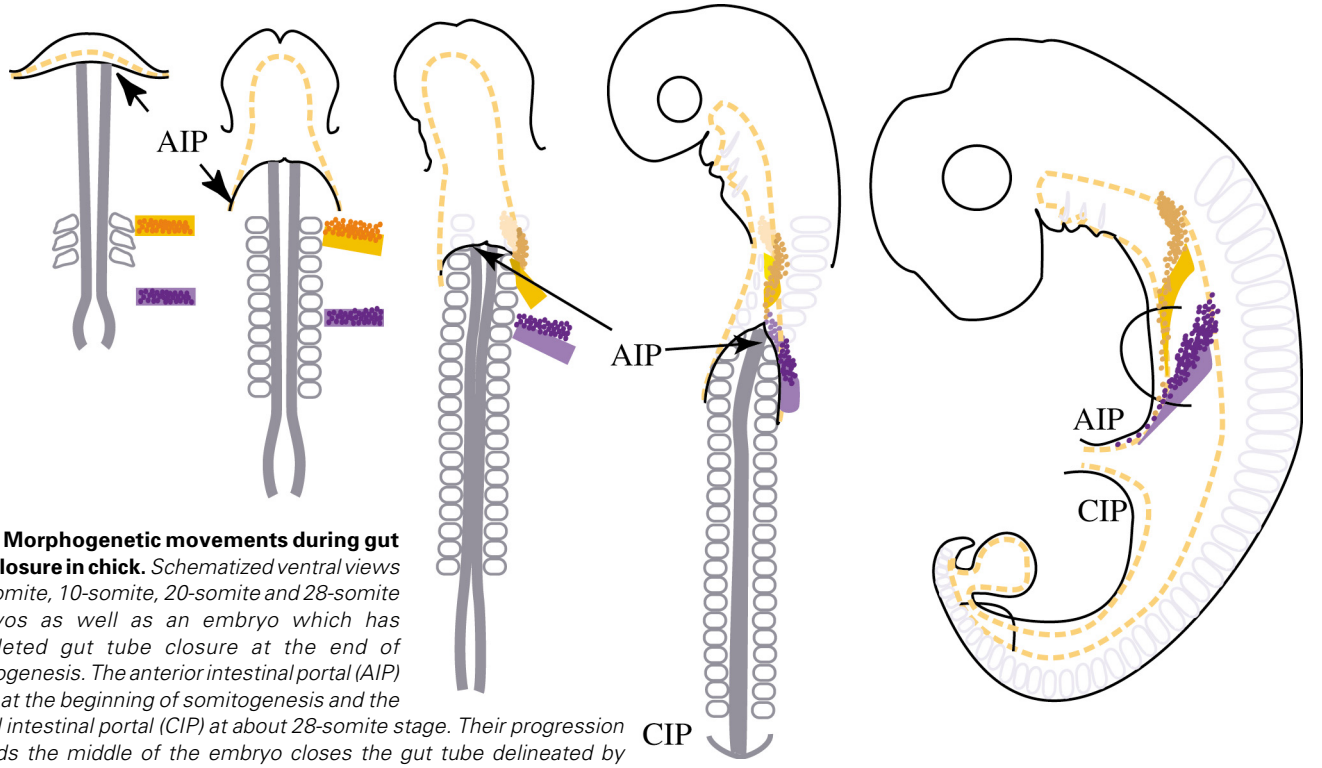

Fig. 3. Morphogenetic movements during gut tube closure in chick. *Schematized ventral views of 3-somite, 10-somite, 20-somite and 28-somite embryos as well as an embryo which has completed gut tube closure at the end of somitogenesis. The anterior intestinal portal (AIP) forms at the beginning of somitogenesis and the caudal intestinal portal (CIP) at about 28-somite stage. Their progression towards the middle of the embryo closes the gut tube delineated by yellow lines. The endoderm at the level of somite 1 is marked by light orange fill. Dark orange dots represent the lateral plate mesoderm from the same level. Somite 6 level is similarly labelled: light purple fill for endoderm and dark purple dots for mesoderm. The enhanced posterior shift of more lateral tissues is seen for endoderm and mesoderm as well as the enhanced posterior shift of endoderm relative to somites and mesoderm. As a result endoderm from a given A-P location contributes to different organs. The same is true for mesoderm. Moreover, endoderm and mesoderm of the same A-P origin end-up in different positions.*

2000) or a few mesenchymal cells were later reported to be present in the culture (Gualdi *et al.*, 1996) (Rossi *et al.*, 2001). Apart from Sumiya and Mizuno (1974) who reported its culture wrapped in vitelline membrane, it is usually cultured in the presence of mesenchyme or in chorioallantoic grafts (Butler, 1935, Le Douarin, 1964a). Heterologous splanchnic mesenchyme generally allows survival and even proper differentiation but limb bud, somitic or cephalic mesoderm are poor substitutes (Le Douarin, 1964a, Le Douarin and Bussonnet, 1966, Le Douarin and Wolff, 1967). The molecular nature of some of the signals produced locally by splanchnic mesoderm has recently been uncovered. Early experiments by Nicole Le Douarin (Le Douarin, 1964a, Le Douarin, 1964b, Le Douarin, 1964c) showed that signals form the mesoderm of the heart are necessary to induce liver development. The presumptive territory of the liver, the AIP, transplanted in other mesodermal territories can develop autonomously after 6 somite-stage but requires the presence of cardiac mesoderm before this stage. As cardiac mesoderm does not induce liver when combined with other endoderm areas, these signals are permissive rather than instructive (Le Douarin, 1975). These properties were recently confirmed in the mouse (Gualdi *et al.*, 1996). It appears that in the mouse, in the absence of cardiac mesoderm, the endoderm of the AIP expresses pancreatic markers (Deutsch *et al.*, 2001). FGF1 and FGF2, which are expressed in the cardiac mesoderm can induce liver markers in the AIP and soluble FGF receptor forms that exert dominant negative effects block liver induction by cardiac mesoderm. It was recently suggested that FGFs sent by the cardiac mesoderm are also required for lung development (Serls *et al.*, 2005) and that they may play an instructive role as different concentrations induce different organ markers. The septum transversum, another lateral plate mesoderm (LPM) derivative, participates in liver induction (Rossi *et al.*, 2001)and later maintenance (Le Douarin, 1963, Le Douarin, 1964a, Le Douarin, 1964c). It produces BMPs. Exposure to noggin, a BMP inhibitor, blocks the convergent ability of cardiac mesoderm and septum transversum to induce liver markers.

At later developmental stages, LPM-derived signals are known to be essential for the maintenance of *Pdx1* expression in the endoderm of the pancreas. Mice mutant in *Isl1* (Ahlgren *et al.*, 1997) and *N-cadherin* (Esni *et al.*, 2001; Edsbagge *et al.,* 2005), two genes expressed in the LPM, do not exhibit LPM convergence around the dorsal pancreas and show a sharp downregulation of *Pdx1* expression as well as an arrest of organ development after initial budding has occurred. *FGF10*, a gene expressed in the mesenchyme around the pancreatic buds from the initiation of budding onward is required for the maintenance of *Pdx1* expression (Bhushan *et al.*, 2001).

In addition to the LPM, two mesodermal components in contact with endoderm provide permissive signals. The notochord, was shown to be required and sufficient for induction of pancreatic endocrine and exocrine markers in the 10-somite-stage presumptive pancreas endoderm (Kim *et al.*, 1997). It sends

permissive signals since it can not induce pancreas markers in more posterior endoderm. FGF2 and activin have been shown to mimic notochord signals but it is not known whether they are involved in vivo (Hebrok *et al.*, 1998). Although ActRIIB$^{-/-}$ and ActRIIA$^{+/-}$B$^{-/-}$ mutants exhibit an anterior transformation of the posterior stomach, it is unclear whether this effect is due to the reception of notochord signals (Kim, 2000). Indeed, the exocrine pancreas is not affected in these mutants although the notochord also controls exocrine pancreas induction. In addition, the LPM around the pancreas is modified in these mutants. It is not known whether the notochord also sends permissive signals in other regions of the gut and plays a general role in dorso-ventral patterning of the endoderm. Blood vessel endothelium is also required for proper differentiation of the dorsal pancreas and liver (Lammert *et al.*, 2001, Matsumoto *et al.*, 2001). In the case of the liver, initial specification happens normally in the absence of endothelial cells but liver cells fail to proliferate and invade septum transversum mesenchyme (Matsumoto *et al.*, 2001). As for the pancreas, the aorta is required for differentiation of endocrine cells and morphogenesis of the dorsal pancreatic bud (Lammert *et al.*, 2001; Yoshitomi and Zaret, 2004). Recent evidence suggests that the vitteline veins which are close to the ventral pancreatic bud are not required for ventral bud initiation (Yoshitomi and Zaret, 2004). It is possible that this association is also important for other digestive organs which also exhibit an early association with endothelial cells (Matsumoto *et al.*, 2001). It is not clear whether any endothelial cells have this inductive capacity.

Lateral plate mesoderm located at different A-P positions sends different instructive information to endoderm

In *Drosophila*, the endoderm (midgut) is patterned by regionalized signals originating from the mesoderm (Bienz, 1994, Bienz, 1997). In Vertebrates, there is a long lasting cross talk between endoderm and mesoderm that can be illustrated on the example of the liver and heart. It has been observed very early that these two organs develop together (Hunt, 1932, Willier and Rawles, 1931). Soon after gastrulation anterior endoderm but not posterior endoderm sends signals that induce cardiomyocytes in mesodermal cells that exit the streak (Schultheiss *et al.*, 1997, Schultheiss *et al.*, 1995). Wnt-inhibitors expressed in anterior endoderm block the heart-repressing activity of posterior Wnts (Marvin *et al.*, 2001, Tzahor and Lassar, 2001; Foley and Mercola, 2005; Schneider and Mercola, 2001). At early somite stages, the heart signals back to endoderm to induce hepatocytes as described above. Later, the septum transversum is required for liver outgrowth. This and other examples lead to the conclusion that positional information in the digestive tract is neither carried by endoderm nor mesoderm but that both carry partial information which varies with time (Yasugi, 1993).

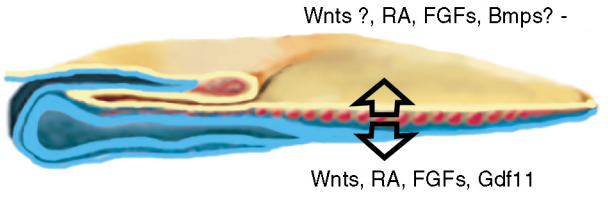

Fig. 4. The mesoderm coordinates A-P patterning in the three germ layers. *A-P patterning of endoderm and neurectoderm is under the control of mesodermal signals. RA and FGFs pattern the two germ layers. Although Wnts play a role in A-P patterning of the neural tube questions remain about the time of action and about its role in endoderm. TGF β family members are involved in patterning both layers but the mechanism and precise members involved need to be better characterized.*

It is still unclear whether there is a general patterning scheme of endoderm that coordinates the whole A-Paxis. We recently showed that at somitic stages, when many positional markers are induced in endoderm, the LPM sends instructive signals to the endoderm layer at least from the duodenum to posterior small intestine (Kumar *et al.*, 2003). In the absence of LPM, the endoderm retains a native endodermal state but does not turn on or retain positional markers (Horb and Slack, 2001, Kumar *et al.*, 2003). It retains its original A-P identity when associated with more anterior endoderm but it is repatterned when associated with more posterior LPM. This is very similar to the patterning mechanism of the neural tube at the same stage as it has been shown that somites and Hensen's node can induce more posterior but not more anterior fates (Grapin-Botton *et al.*, 1997, Itasaki *et al.*, 1996, Liu *et al.*, 2001, Muhr *et al.*, 1997). The somites share with the LPM the ability to induce posterior markers in endoderm (Kumar *et al.*, 2003). This suggests that the mesoderm coordinates the patterning of all three germ layers (Fig. 4). As in the neural tube Hensen's node also sends posteriorizing signals as late as somitic stages, it will be interesting to see whether the node also plays such a late role in endoderm patterning (Liu *et al.*, 2001). Plasticity at equivalent stages had been observed in frogs where stage 22-25 but not stage 28 endoderm looses *Xlhbox8/Pdx1* expression when associated to posterior mesoderm (Zeynali *et al.*, 2000). A gradient of signaling along the A-P axis is compatible with the observations of Nicole Le Douarin who grafted the liver endodermal primordium after 6-somite-stage in splanchnopleura and showed that the more posterior the graft the less liver epithelial cords developed (Le Douarin, 1963, Le Douarin, 1964c). It is also in agrrement with the observation that small intestine endoderm loses very early its ability to be repatterned when associated with mesenchyme from other regions (Andrew and Rawdon, 1990, Gumpel-Pinot *et al.*, 1978, Yasugi, 1993, Yasugi and Mizuno, 1978, Yasugi *et al.*, 1991). It is still unclear whether the posterior dominance of mesoderm observed here holds true along the entire A-P axis as already a few discrepancies are found in the literature. The floor of the pharynx, grafted in the splanchnopleure at similar stages and later found in the small intestinal wall or in the body wall, forms thyroid, thymus, muscular and glandular stomach, liver, exocrine pancreas and intestine (Le Douarin and Bussonnet, 1966, Le Douarin and Wolff, 1967). The anterior marker *Sox2* (Fig. 2) is induced in small intestine until E4 (equivalent to 11.5 dpc in the mouse) (Ishii *et al.*, 1998).

Plasticity seems to be lost long before birth. Ishii and colleagues showed that the stomach epithelium is posteriorized by small intestine epithelium until E4 (equivalent to 11.5 dpc in the mouse) but at E6 (about 12.5dpc) although signals are still produced by the mesenchyme, the endoderm has lost its ability to express the intestinal markers *CdxA* and sucrase (Ishii *et al.*, 1997, Ishii *et al.*, 1998) (Haffen *et al.*, 1982, Ishizuya-Oka and Mizuno, 1984). In agreement, 14.5dpc stomach or lung epithelium do not appear to be influenced by small intestine mesenchyme (Duluc *et al.*, 1994).

The mesenchymal signals seem to perdure after the loss of plasticity (Ishii *et al.*, 1997). It is not always clear whether the signals identified pertain to general A-P patterning or to local events. For instance, stomach glands can develop in many epithelia including posterior small intestine until late stages (E9). In this case the glandular structure forms but it does not express pepsinogen and it retains sucrase (Takiguchi-Hayashi and Yasugi, 1990, Yasugi *et al.*, 1985). Information to generate the structure is in the mesenchyme but A-P information may reside in the epithelium. In this case, the muscular stomach mesenchyme mixed 1:9 with glandular stomach mesenchyme blocks the ability to induce glands even if muscular stomach clls are far from the epithelium, thus suggesting local repressive secreted signals acting at a distance (Urase and Yasugi, 1993).

A-P identity in paraxial mesoderm is coupled with segmentation and determined once somites are formed (Dubrulle *et al.*, 2001, Kieny *et al.*, 1972, Nowicki and Burke, 2000, Zakany *et al.*, 2001). There is probably later plasticity of A-P identity in somatopleure since when an additional limb is induced by application of FGF-loaded beads between the wing and leg *Hox* gene expression in somatopleure is either shifted posteriorly to ressemble the wing pattern or shifted anteriorly to match the leg pattern (Cohn *et al.*, 1997). Moreover the LPM environment changes A-P identity in isolated somitic cells that migrate into the LPM to form limb muscle (Nowicki and Burke, 2000). Nothing such is known about the splanchnopleure.

Molecular basis of endoderm A-P patterning

Retinoic acid induces posterior character in endoderm as in neurectoderm

In the neural tube, FGFs (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Mathis, *et al.*, 2001; Storey, *et al.*, 1998), Wnts (Erter, *et al.*, 2001; Mcgrew, *et al.*, 1997; Nordstrom, *et al.*, 2002) and retinoic acid (RA) (Maden, 1999) are known to induce posterior fates in a graded manner (Bel-Vialar, *et al.*, 2002; Liu, *et al.*, 2001). More recently, the TGFβ family Growth/Differentiation Factor GDF11, was also proposed to be responsible for the induction of the most posterior fates, in conjunction with FGFs (Liu, *et al.*, 2001; Mcpherron, *et al.*, 1999).

As in the neural tube, RA has posteriorizing effects on the endoderm. At gastrulation stages in the chick it is able to repress the anterior marker *Otx2* (Bally-Cuif, *et al.*, 1995). The role of RA in endoderm organ formation has been reported in Zebrafish, *Xenopus*, mouse and chick (Chen, *et al.*, 2004; Kumar, *et al.*, 2003; Molotkov, *et al.*, 2005; Stafford, *et al.*, 2004; Stafford and Prince, 2002). These publications focused largely on the pancreas and showed that retinoic acid is important at gastrulation stages to induce this organ and to control the balance between endocrine and exocrine cells. Although analyzed in less detail, two of these publications suggest that RA controls endoderm organ position along the A-P axis as it does in the neural tube. In Zebrafish, RA induces an anterior shift in endoderm gene expression throughout the foregut (down to duodenum/pancreas) and blocking the pathway prevents induction of the most posterior fates (Stafford and Prince, 2002). In chick, Kumar *et al.* (Kumar, *et al.*, 2003) observed that RA induces *Pdx1*, a pancreato-duodenal marker in the stomach/esophagus, but also extends expression of *CdxA*, a small and large intestine marker as anterior as the esophagus. However, the effects described in the chick occur at much later stages, when somites have formed. Although the requirement for RA in endoderm patterning has not been addressed in this study, it is clear that endoderm still responds to RA at 10-somite-stage unlike in fish and *Xenopus*. Many A-P markers of endoderm are induced at this stage. It also controls A-P patterning of endoderm in *Amphioxus* (Escriva, *et al.*, 2002; Schubert, *et al.*, 2005), but these studies contradict previous findings showing that in *Xenopus*, RA does not affect the expression of the pancreatic marker *Pdx1* after treatment of whole embryos at stage 12 and 25 (Zeynali and Dixon, 1998). The late action of RA in chick is consistent with a posterior to anterior gradient of RA proposed to be present in chick LPM at the 10-somite stage (Swindell, *et al.*, 1999). Indeed, Raldh2, an enzyme responsible for RA synthesis is expressed in the streak and later in somites and trunk LPM, tissues that have a posteriorizing activity. RA is thus synthesized only in the trunk. Cyp26, an enzyme responsible for RA degradation and often expressed in tissues that respond to RA is in lateral endoderm. Its expression forms a gradient at stage 14, with high expression at the level of the first somite and going down towards somite 15. The amount of signal received posteriorly may thus be higher due to lower degradation rates. (Blentic, *et al.*, 2003). It is not clear whether RA patterns the endoderm all along the axis. In the neural tube RA only plays a role in regulating genes from the hindbrain (pharynx level) to the cervical level (Hoxc-6). It downregulates more posterior *Hox* genes *in vivo* and *in vitro* (Bel-Vialar, *et al.*, 2002; Liu, *et al.*, 2001; Simeone, *et al.*, 1991). In endoderm, RA appears to induce the caudal homologue *CdxA* in the chick and not to affect it in Zebrafish (Kumar, *et al.*, 2003; Stafford and Prince, 2002). In each of these cases it is unclear if RA is acting directly upon the endoderm, or indirectly via the mesoderm. The best evidence for direct action of RA upon the endoderm has been provided by investigating the *Hoxb1* promoter, which is regulated by RA in endoderm and ectoderm through two different promoter elements (Huang, *et al.*, 2002; Huang, *et al.*, 1998). No such elements have been identified in the *Pdx1* or *CdxA/1* promoters. This pathway may interact with the FGF pathway in endoderm during gastrulation as the FGF pathway regulates the expression of RA receptors and of enzymes that control RA synthesis and degradation (Shiotsugu, *et al.*, 2004) and conversely RA signaling regulates FGF receptor expression.

FGFs and Wnts, posterior inducers in the nervous system may act on endoderm patterning

In the nervous system at somite stages, RA is responsible for pattern formation at the level of the hindbrain and cervical level. More posteriorly, *Hox* gene expression is induced by FGFs (mainly 2 and 4 and much less efficiently 8) that act partially through Cdxs (Bel-Vialar *et al.*, 2002; Charite *et al.*, 1998; Liu, *et al.*, 2001). In mouse endoderm, FGF4 (but not FGF2, 5 or 8) applied to anterior endoderm of late gastrulas (7.5 dpc) induces markers that are characteristic for the posterior half of the embryo. FGF4 is expressed in the posterior primitive streak and later in the tail bud (Niswander and Martin, 1992). Embryos knockout for *Fgf4* arrest before gastrulation (Feldman *et al.*, 1995) and therefore the requirement for FGF4 in A-P patterning has not been tested. As in the nervous system, FGF2 and 4 are the most potent posterior

inducers. Our recent experiments suggest that FGFs also function later to pattern the mid-hindgut (Dessimoz et al., unpublished). FGFs may have a permissive action and maintain cells in a state of responsiveness to other signals (Mathis *et al.*, 2001; Nordstrom *et al.*, 2002). FGF response elements that bind ets transcription factors (downstream targets of FGFs) have been recently evidenced in *Xcad3* (*CdxB*) promoter in *Xenopus*. *CdxB* may thus be a direct endoderm target of FGF signaling (Haremaki *et al.*, 2003). *Pdx1* may also be a direct target of the FGF4 signaling.

Wnts, in the presence of FGFs, play a role in A-P patterning of the nervous system (Holland, 2002; Mcgrew *et al.*, 1997; Nordstrom *et al.*, 2002). Wnt8 is expressed in the caudal paraxial mesoderm in gastrulating and headfold stage embryos and activates posterior markers in a graded manner on isolated neural tube. It is not clear whether it keeps signaling at later stages. Wnt3A is expressed in the tail bud, as is wnt8 in a subset of Vertebrates (Holland, 2002). *Cdx1/A* is a direct target of canonical Wnt signaling that is important for induction of this gene in primitive streak, somites (wnt3A) and possibly endoderm (Ikeya and Takada, 2001; Lickert *et al.*, 2000; Lickert and Kemler 2002). There may be a feedback loop as *CdxB*, a chick caudal gene can induce *Wnt8c* when misexpressed in the heart (Ehrman and Yutzey, 2001). Since *Cdx1* is induced late during development in the endoderm (14 dpc) it would be interesting to know whether induction of earlier endodermal markers is also dependant on Wnt signaling. None of the isolated *Cdx1* promoter elements including those with Wnt pathway target sites drive endodermal expression (Lickert and Kemler, 2002).

It is of interest that in *C. elegans*, all but one division in the E lineage occur along the A-P axis. These divisions are asymmetric such that only the anterior cell inherits the Tcf-related protein POP-1 (Lin *et al.*, 1998). In the first asymetric cleavage of EMS that generates the endodermal precursor E posteriorly, this is due to the production of the MOM-2 Wnt ligand by the P2 posterior cell (Lin *et al.*, 1998). Later divisions require MOM-5/Frizzled but not MOM-2/Wnt (Park and Priess, 2003). In *Drosophila*, Wingless is secreted by the mesoderm surrounding the endoderm where it induces *labial* and later copper cells at low levels while closer to its site of production it represses *labial* and induces the differentiation of large flat cells (Hoppler and Bienz, 1995).

A role for BMPs in endoderm A-P patterning: differences between endoderm and neurectoderm?

In *Drosophila*, *Hox* genes are regionally expressed in the mesoderm and control the local secretion of Decapentaplegic (Dpp). Dpp is expressed in a specific band in the midgut mesoderm, next to Wg. It regulates *Hox* gene expression in mesoderm and endoderm (Immergluck *et al.*, 1990, Staehling-Hampton and Hoffmann, 1994, Staehling-Hampton *et al.*, 1994, Tremml and Bienz, 1989)(Tremml and Bienz, 1989; Immergluck *et al.*,1990; (Panganiban *et al.*, 1990). Expression of labial in endoderm depends on cooperation of Hox binding sites that bind labial and DPP responsive elements (Marty *et al.*, 2001). LEF1 and DPP/mad binding sites in ultrabithorax promoter directly control this gene (Riese *et al.*, 1997).

In Vertebrates, we showed that BMP2, 4 and 7 as well as activin expand *Pdx1* and *CdxA* expression anteriorly in endoderm (Kumar *et al.*, 2003). In addition noggin and follistatin block induction of posterior genes elicited by LPM, suggesting that a BMP rather than activin is implicated (activin is not blocked by noggin). As we have discussed that during the morphogenesis of the gut more lateral endoderm eventually contributes to more posterior tissue, it is possible that the effect of BMPs is a lateral transformation. In Zebrafish, Tiso *et al.* (Tiso *et al.*, 2002) have demonstrated that *swirl* (*BMP2b*) mutants have a general reduction in posterior endoderm identities. On the contrary, *chordino* mutants have expanded posterior gut and reduced anterior digestive tract. Although the effect of BMP2b appears to be very early in fish since the expression of the *hairy* gene *her5* in anterior endoderm was affected in *swirl* and *chordino* mutants at 80% epiboly (late gastrulation), it has been shown that activin does not posteriorize 7.5 dpc mouse endoderm (gastrulation). There may be a difference of timing in A-P patterning in these two species as seen before for retinoic acid (Wells and Melton, 2000). The two first publications show that these ligands are able to induce gene expression characteristic of multiple regions along the A-P axis and are thus likely to act in a graded manner although this has not been demonstrated yet. One would then expect to see a graded expression of one or several BMPs or activin along the A-P axis at the stages studied here. Indeed, the LPM adjacent to the presomitic mesoderm strongly expresses BMP4 with expression becoming progressively weaker in the LPM adjacent to fully-segmented somites (Pourquie *et al.*, 1996, Reshef *et al.*, 1998, Schultheiss and Lassar, 1997) (our unpublished results). In addition, the expression of multiple BMPs has been reported in the mesenchyme surrounding the endoderm (Roberts *et al.*, 1995, Smith *et al.*, 2000, Solloway and Robertson, 1999, Winnier *et al.*, 1995) while the BMP receptors *BMPRIA* and *BMPRII* are expressed in endoderm (Mishina *et al.*, 1995, Roelen *et al.*, 1997). Activin receptor II inactivation in the mouse reveals a requirement for these receptors in A-P patterning in the foregut (Kim *et al.*, 2000). Since Gdf11 plays a role in posterior gene induction in the neural plate it would be interesting to see if a GDF is involved in posteriorization of endoderm (Liu *et al.*, 2001, McPherron *et al.*, 1999).

Roberts *et al.* (Roberts *et al.*, 1995) showed that Shh induces *Hox* genes in the posterior hindgut. Shh is initially expressed in the endoderm of the CIP when the gut folds and later on spreads to most of the gut tube. Precocious activation of Shh in the gut mesoderm induces an anterior shift of *Hoxd11* and *Hoxd13* expression in mesoderm. This shift is limited since regions anterior to the yolk stalk are not competent to express these genes at this stage. It is not clear how Shh action is restricted as this gene is expressed in the AIP where it does not induce posterior *Hox* genes. Since Shh also induces BMPs in the mesoderm, the ability of Shh to induce posterior genes may be relayed by BMPs.

Conclusion

Beyond characterizing the interactions between the retinoic acid, Wnt, FGF and BMP pathways in endoderm patterning, new directions of research may go towards further characterization of endoderm-mesoderm interactions in light of the sliding movements observed by Nicole Le Douarin. Fate mapping the AIP with modern techniques should in particular solve the question as to whether single cells of the AIP can contribute to different organs.

The control of A-P identity in endodermal cells is important not only during development but must be maintained in the adult. The

loss of identity leads to metaplasia which often evolve in neoplasia. Mice which have lost one copy of *Cdx2* in their genome develop adenomatous polyps, mostly in the proximal colon (Beck *et al.*, 2003, Beck *et al.*, 1999, Chawengsaksophak *et al.*, 1997). These polyps contain areas were *Cdx2* is not expressed although there is no loss of heterozygosity. These *Cdx2*-negative domains show a continuous sequence of all digestive tract epithelia from colon to esophagus suggesting a gradient of signaling. Dysplasia is observed in 20% of lesions. Although *Cdx2* expression is often lost in colon cancer, it is rarely mutated (da Costa *et al.*, 1999, Mallo *et al.*, 1997, Yagi *et al.*, 1999). Another example of induced metaplasia mimics a human affection. When *Cdx2* is ectopically activated in the stomach, intestinal metaplasia is induced (Silberg *et al.*, 2002). *Cdx2* expression is observed in most gastric intestinal metaplasia and gastric carcinoma (Almeida *et al.*, 2003, Bai *et al.*, 2002, Seno *et al.*, 2002). In the esophagus, 10% of Barrett metaplasia, which consist in gastric tissue in the esophagus, evolve in esophagal cancer (OMIM 109350). The role of *Cdx* up- or down-regulation in the evolution to neoplasia and the factors that cause it are interesting questions for the future. In particular, since the mesenchyme controls *Cdx* expression during development, the role of the stroma would be worth investigating.

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