Endoderm specification

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Abstract

In this chapter I focus on the emergence of endoderm, the origin of these cells and their organization in space. I also discuss the molecular events that lead to endoderm formation and how endoderm can be molecularly defined. Although the molecular control of endoderm formation has initially been deciphered using Xenopus, Zebrafish, sea urchin and several other species many molecular switches have been confirmed in mice. This article preferentially cites references in the mouse model system but data from other model organisms are used when they provide important information missing in mice. Extensive references to other species can be found in Grapin-Botton; Constam, 2007 and Stainier, 2002. This article presents endoderm engineering from ES cells and provides molecular triggers and landmarks that may be used for optimized engineering based on normal development. Due to the similarity of markers between definitive and extraembryonic endoderm and the recent discovery that visceral endoderm can contribute to the digestrive tract, the generation of these lineages is also discussed (Kwon et al., 2008). Although endoderm stem cells, that is stem cells endowed with the ability to give rise to all endodermal derivatives but not ectoderm or mesoderm, have not been reported yet, there are stem cells in specific endodermal organs which will be discussed in the following chapters.

1. Introduction

The endoderm is classically defined as the inner germ layer of the embryo. The main derivative is the epithelial outlining of the digestive tract but it does also contribute to many other organs detailed in Figure 1. However, the terminology in Amniotes is confusing as different types of cells that contribute to extraembryonic structures have

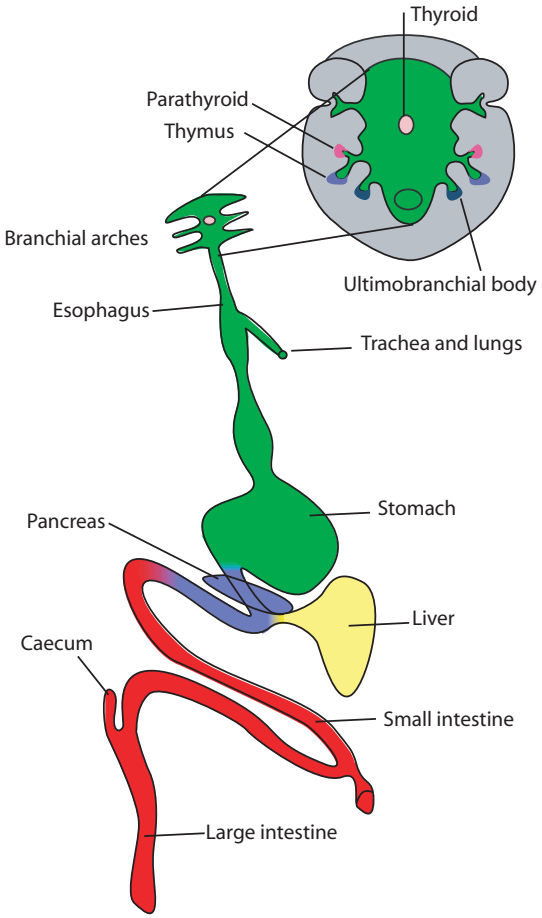

Figure 1. Endoderm derivatives.

also been called endoderm, such as the primitive (PrE), visceral (VE) and parietal endoderm (PE). Figure 2 depicts the location and lineage of the different types of endoderm. The fact that these extraembryonic structures share many molecular markers with definitive endoderm adds to the confusion.

2. Cellular aspects of endoderm emergence

2.1. Emergence of definitive endoderm at gastrulation

Definitive endoderm in Amniotes arises at the time of gastrulation, during which endoderm precursors initially located in the epiblast ingress in the anterior primitive streak (see Figure 1). Definitive endoderm cells egress from the primitive streak and insert into the visceral endoderm. VE forms in majority extraembryonic tissues but also contributes some cells to the gut tube (Kwon et al., 2008). Recent obvservations suggest that cells originating from the epiblast visceral endoderm intermingle between VE cells (Kwon et al., 2008) rather than displacing it as a sheet to anterior and lateral regions of the conceptus (Poelmann, 1981)(Lawson et al., 1986; Tam and Beddington, 1992; Tam et al., 2004; Tam et al., 1993). Definitive endoderm movement is accompanied by epithelial-mesenchymal transition and requires *Snail* or the related gene *Slug* that transiently repress E-cadherin (Blanco et al., 2007; Nakaya et al., 2008; Thisse et al., 1995). The directionality of movement is controlled by mesoderm-derived Sdf1/Cxcl12b acting on Cxcr4-expressing endoderm in zebrafish and Xenopus (Fukui et al., 2007; Mizoguchi et al., 2008; Nair and Schilling, 2008). Mosaic genetic modifications in Zebrafish have shown that at least in this model, endoderm gastrulation is a combination of active movements of cells and passive movements whereby a cell is mobilized by its neighbours (Carmany-Rampey and Schier, 2001; Pezeron et al., 2008). Sub-populations of animal pole cells forced to express Sox17- an inducer of endoderm- either migrate towards the endodermal layer or die. These observations suggest that there is a feed-back check on the matching between the differentiation status of a cell and its environment (Clements and Woodland, 2000).

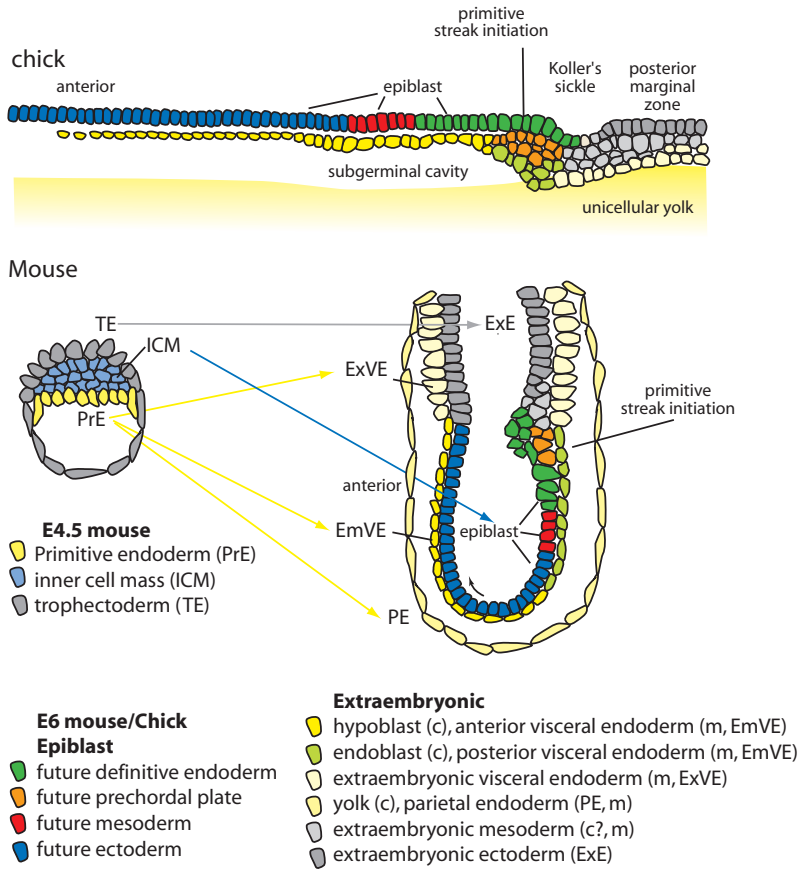

Figure 2. Organization of endodermal progenitors and extraembryonic endoderm at onset of gastrulation in chick and mouse embryos. Extraembryonic endoderm lineages and the fate of epiblast cells are color-coded. The territories of prospective endoderm, mesoderm and neurectoderm are separated by sharp boundaries only for the sake of simplification. In the mouse, posterior VE is speculated to be the equivalent of the chick endoblast due to its analogous position. Likewise in the proximal epiblast, prechordal plate progenitor cells are predicted to reside in between definitive endoderm precursors and posterior VE in analogy to chick, even though the limited resolution of current fate maps cannot distinguish two separate populations in the mouse epiblast.

The latter considerations, in the context of ES cell cultures may allow endodermal cells to aggregate in a heterogeneous culture or die depending on their neighbours.

2.2. Timing of endoderm specification

In mouse and chick, heterotopic grafting experiments have shown that determination to form endoderm occurs after the cells have left the streak (Kimura et al., 2006; Kinder et al., 2001). Whether the EMT is crucial for endoderm differentiation is unclear at the moment but the ability of endoderm to differentiate prior to gastrulation in several species argues against this hypothesis (Laufer et al., 1980; Leung et al., 1999; Priess and Thomson, 1987; Schroeder and McGhee, 1998). Nevertheless in Amniotes, cells are exposed to signalling centers during their migration. In chick, endoderm progenitors in Koller's sickle undergoing their characteristic 'Polonaise movement' are thought to become specified as they receive signals from the posterior marginal zone which activate the Nodal signaling pathway. These signals include Wnt ligands (Skromne and Stern, 2001). At a later stage, mesoderm and endoderm in passing may receive instructive patterning signals from the node (Brennan et al., 2002; Pagan-Westphal and Tabin, 1998). Such signaling centers are formed in ES cell cultures aggregated into embryoid bodies and most likely in dense monolayer cultures (Leahy et al., 1999). By sensing their position relative to signaling center, moving cells thus might coordinate their differentiation.

2.3. Mesendoderm

With the notable exception of sea urchin, most species initially segregate ectoderm precursors from progenitors that give rise to endoderm and mesoderm. In *C. elegans*, sea urchin and zebrafish mesoderm and endoderm derive from bipotential progenitors (Rodaway and Patient, 2001). In Amniotes a similar mesendoderm population has been postulated based on coexpression of endoderm and mesoderm markers in the anterior streak (Rodaway et al., 1999), and the observation that certain signalling cascades induce both types of cells (Lemaire et al., 1998; Reiter et al., 1999; Rodaway and Patient, 2001). In space, endodermal/mesendodermal progenitors tend to be located in the anterior streak whereas mesodermal progenitors extend to the posterior streak. However, single cell lineage tracing has never formally demonstrated the existence of bipotential cells in Aminotes.

3. Molecular control of definitive endoderm formation

A comprehensive analysis of the regulatory gene network responsible for endoderm differentiation has been carried out in sea urchin and largely corroborated in *Xenopus* (Davidson et al., 2002; Davidson et al., 2002; Loose and Patient, 2004). Experiments in Zebrafish also pioneered the molecular elucidation of endoderm formation (Stainier, 2002). In non-Amniotes endoderm is initially induced by maternal proteins which will not be discussed here (β-catenin, VegT, Otx). They do not have a maternal activity in Amniotes although β-catenin and eomesodermin (a T-box gene like VegT) have a zygotic activity in endoderm induction. In Amniotes endoderm is initially induced by secreted factors.

3.1. The TGFβs Nodal, Gdf1 and Gdf3 are endoderm inducers in vertebrates

Insights into the inductive mechanisms underlying endoderm formation in vertebrates initially came from studies in *Xenopus* using a dominant-negative activin receptor which blocks secreted TGFß-related activities including Activin, Vg1, and *Xenopus* nodal-related proteins (Xnr). Vegetal pole endoderm explants of embryos injected with this construct express mesodermal and ectodermal marker genes at the expense of endoderm (Henry et al., 1996). This led to the idea that Vg1 and/or Xnr's are endogenous endoderm inducers. Further analysis of Nodal functions in *Xenopus* and zebrafish confirmed that mesodermal and endodermal cell fates are specified by different levels of Nodal signaling (Agius et al., 2000; Dougan et al., 2003; Schier et al., 1997; Thisse et al., 2000). Also in the mouse, Nodal induces both mesoderm and endoderm (Brennan et al., 2001; Conlon et al., 1994; Zhou et al., 1993), but endoderm populations appear to be selectively lost in embryos carrying a hypomorphic *Nodal* allele, mutations in Nodal proteolytic activation site or gradual reductions in the gene dosage of Smad 2 and 3 (Ben-Haim et al., 2006; Dunn et al., 2004; Liu et al., 2004; Lowe et al., 2001; Norris et al., 2002). Conversely, targeted inactivation of the Nodal antagonist Lefty2 leads to excess endoderm formation (Meno et al., 1999). Studies in zebrafish suggest that Nodal proteins establish a morphogen gradient to pattern the marginal zone along the animal-vegetal axis, with peak levels specifying blastomeres closest to the margin to form endoderm. In contrast, cells farther away from the Nodal source respond by expressing mesodermal genes, presumably because they are exposed to lower concentrations of Nodal, and/or a shorter duration of signaling (Chen and Schier, 2001). The regulation of Nodal expression is discussed in the next chapter (see Figure 3). Homologues to the second activin receptor binding protein, Vg1, have been identified in

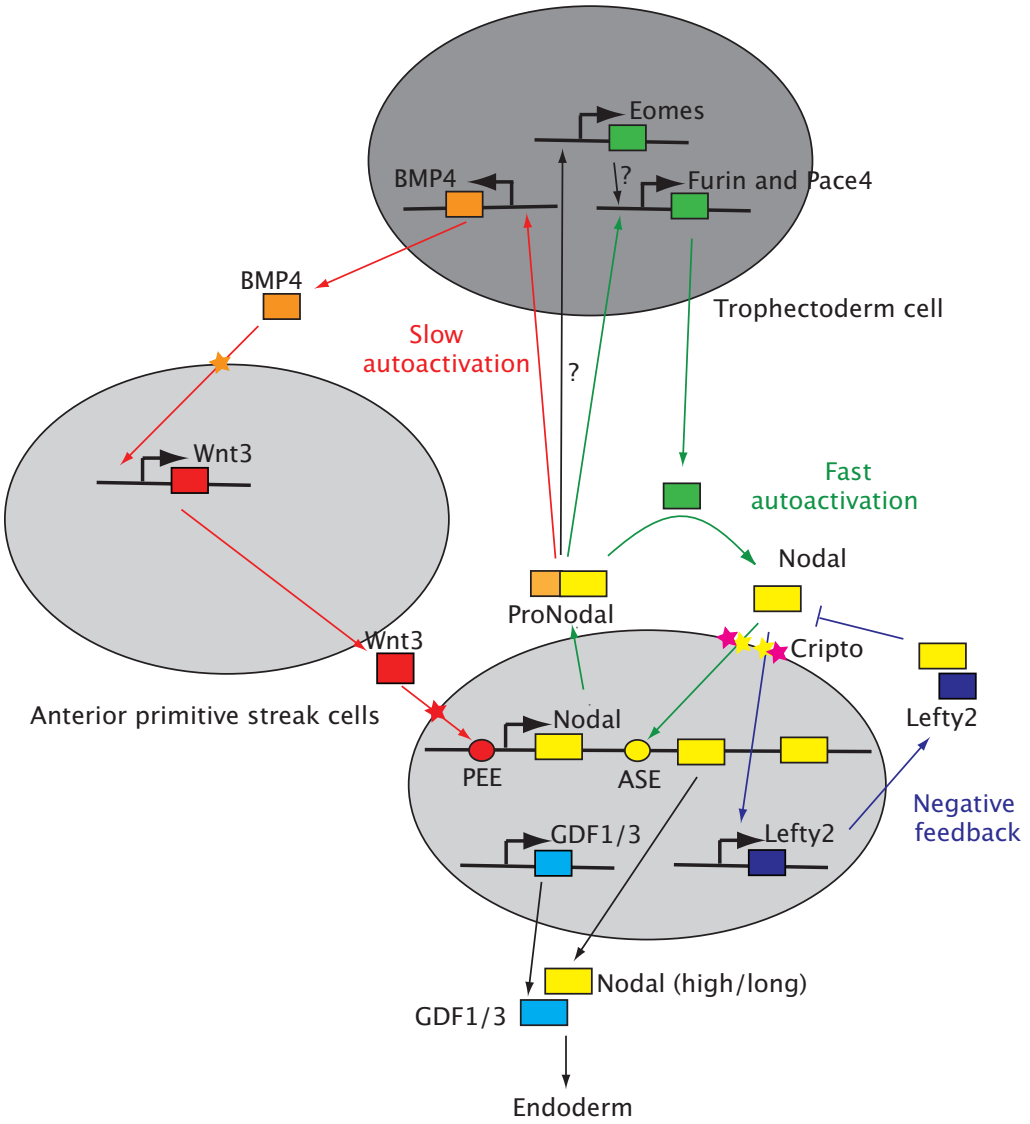

Figure 3. Feedback loops of secreted factors induce endoderm. Two positive feedback loops (in green and red) involving crosstalk between epiblast cells at the site of future primitive streak and trophectoderm allow to generate the high and long lasting levels of Nodal that induce endoderm. GDF1/3 act as Nodal co-factors in endoderm induction. A negative feedback loop (in blue) involving Lefty2 shuts down signalling activity to limit the amount of endoderm produced. Stars represent receptors. PEE ans ASE are characterized promoter elements. Questionmarks refer to putative interactions

zebrafish, chick and more recently in mouse where the more distant Gdf1 and 3 recapitulate Vg1 activity (Dohrmann et al., 1996; Helde and Grunwald, 1993; Seleiro et al., 1996; Shah et al., 1997: Andersson et al., 2007; Bertocchini et al., 2004; Skromne and Stern, 2001). *Gdf1* and *3* are expressed in the node like Nodal and recent experiments show that Gdf1 potentiates Nodal activity by forming heterodimers that signal at a longer range (Tanaka et al., 2007; see Figure 3).

3.2. Nodal expression is induced by the canonical Wnt pathway and positive feedback signaling

Among the signals which activate *Nodal* expression is the Wnt pathway. Mouse embryos lacking Nodal, or ß-catenin fail to form a primitive streak (Conlon et al., 1994; Huelsken et al., 2000), suggesting that the canonical Wnt pathway and Nodal act in synergy to specify definitive endoderm. Several *Wnt* genes are expressed before and during gastrulation (Kemp et al., 2005). Analysis of Wnt null alleles demonstrates that germ layer formation and expression of mesendoderm markers in the mouse depends on *Wnt3* (Liu et al., 1999). At the onset of gastrulation, *Wnt3* is initially expressed in the posterior visceral endoderm and proximal epiblast region shortly before prospective

mesendoderm cells begin to ingress into the primitive streak. *Wnt3* and *Nodal* mutually activate each other (Ben-Haim et al., 2006; Brennan et al., 2001; Liu et al., 1999). Thus, the canonical Wnt pathway may promote endoderm formation in mammals primarily by locally stimulating Nodal feedback signalling (see Figure 3).

Residual Nodal signaling in *Wnt3* and *ß-catenin* mutants indicates that Nodal expression is regulated by additional signals. Peak levels of *Nodal* expression depend on an autoregulatory loop mediated by the binding of FoxH1 on a FAST binding site in the *Nodal* regulatory region (Hoodless et al., 2001; Norris et al., 2002; Yamamoto et al., 2001). This autoregulation is potentiated by cripto, an EGF-CFC family GPI-anchored glycoprotein, that can directly associate with Nodal and its signaling receptor Alk4 (Reissmann et al., 2001; Yeo and Whitman, 2001). Although Cripto promotes mesendoderm formation primarily by stimulating Nodal signalling, recent data suggests that Cripto binds Wnt11 and stimulates activation of ß-catenin (Tao et al., 2005).

Similar to other TGFß family members, Nodal is derived from a precursor protein by redundant proteolytic activities of the subtilisin-like proprotein convertases Furin or Pace4 (Constam and Robertson, 1999). Embryo explant experiments and analysis of Furin$^{-/-}$; Pace4$^{-/-}$ double mutants suggested that Nodal processing is essential to stimulate autoinduction early after implantation, but that uncleaved Nodal during gastrulation may activate at least a subset of mesodermal markers (Beck et al., 2002; Mesnard et al., 2006). Endoderm formation depends on two sequential positive feedback loops mediated by Cripto and Bmp4/Wnt3 that are activated by mature or uncleaved Nodal, respectively, to sustain Nodal signaling from implantation throughout gastrulation (Ben-Haim et al., 2006). According to a recent mathematical model as well as evidence in Zebrafish, the choice between mesodermal and mesendodermal fates depends on how long a particular cell and its ancestors have been exposed to Nodal and its effectors, rather than a concentration gradient (Ben-Haim et al., 2006; Hagos and Dougan, 2007). Recent experiments have demonstrated that the T-box gene Eomesodermin, expressed in the trophectoderm like Furin and Pace 4, synergizes with Nodal (Arnold et al., 2008). It may participate in one of the feed-back loops (see Figure 3).

This important role of Nodal emerged recently as it has different functions in Echinoderms, Ascidian and possibly Amphioxus (Duboc et al., 2004; Hudson and Yasuo, 2006; Yu et al., 2002). The role of Nodal in endoderm and mesoderm induction in vertebrates may have derived from its original function in dorso-ventral axis specification. The role of β-catenin and Wnt signalling in endoderm induction is prominent in C.elegans, Echinoderms and all vertebrates (Reviewed in (Grapin-Botton and Constam, 2007). Nodal autoregulation and induction by Wnts/β-catenin functions in echinoderms and must predate chordates. Clearance of FoxQ2, a Nodal repressor, by β-catenin maintains Nodal expression in Sea Urchin (Yaguchi et al., 2008). Although FoxQ2 identification in Vertebrates is awaiting, its expression in Chordates suggests that this function may be evolutionary conserved (Yu et al., 2003).

3.3. GATA factors are expressed in mesendoderm and required for endoderm differentiation

Several transcription factors are acting downstream of secreted factors responsible for endoderm induction in a network tentatively represented in Figure 4 although the direct regulatory mechanisms are still largely unknown. Several Gata family members are acting downstream of Nodal although the proteins leading to Gata activation are different in several model organisms (Grapin-Botton and Constam, 2007).

Forkhead transcription factors of the FoxA family and GATA factors are key players of the endodermal network of transcription factors in all triploblasts studied so far. Several family members are expressed in endoderm or mesoderm in most species. Inactivation of Serpent, one of the Drosophila Gatas precludes endoderm formation (Rehorn et al., 1996; Reuter, 1994). Among the 6 vertebrate *Gata* genes, *Gata4*, *5* and *6* play partially redundant roles in endoderm development. Zebrafish *faust* mutants lacking *Gata5*, contain about 60% of the wild type number of endodermal cells (Reiter et al., 1999). *Gata4* (Jacobsen et al., 2002; Soudais et al., 1995) and *6* (Koutsourakis et al., 1999; Morrisey et al., 1998), but not *Gata5* (Molkentin et al., 2000) knockout mice show impaired visceral and definitive endoderm development. In *Xenopus*, *Gata4, 5* and *6* are expressed in endoderm and convert ectomesoderm into endoderm in a redundant manner (Afouda et al., 2005; Gao et al., 1998; Jiang and Evans, 1996; Weber et al., 2000). In vertebrates, GATA factors have been shown to activate genes involved in adult endodermal cell function (Intestinal fatty acid binding protein, hepatic nuclear factor 4/HNF4, gastric H+/K+ ATPase) and in some cases such as albumin bind their promoter (Bossard and Zaret, 1998; Gao et al., 1998; Maeda et al., 1996; Morrisey et al., 1998).

3.4. Forkhead factor expression and requirement for endoderm differentiation

Forkhead genes of the FoxA class are also expressed in endoderm all triploblasts (Harada et al., 1996; Ang et al., 1993; Horner et al., 1998; Kaestner et al., 2000; Kalb et al., 1998; Monaghan et al., 1993; Odenthal and

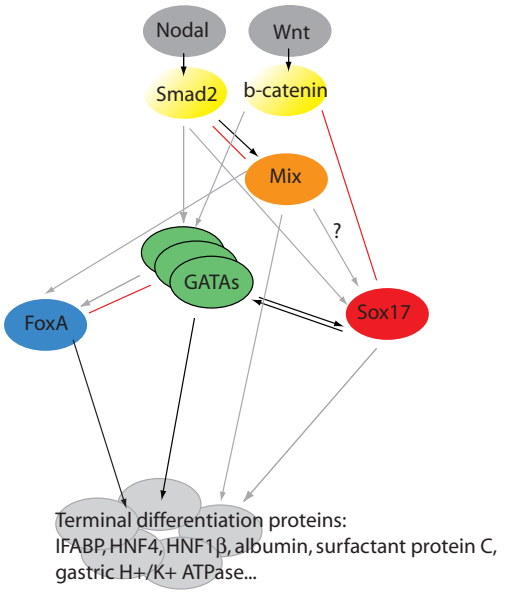

Figure 4. Transcription factor network in endoderm induction. Grey arrows show inductions. Black is used when direct regulation was demonstrated. Red lines show molecular interactions. Some of the regulations have not been demonstrated in mice and may come from Zebrafish or Xenopus. Mix, Sox17 and Gatas each regulate a subset of differentiation genes (Sinner et al., 2006).

Nusslein-Volhard, 1998; Sasaki and Hogan, 1993; Schier et al., 1997; Strahle et al., 1993; Weigel et al., 1989). Their inactivation perturbs but does not abolish endoderm development. Interestingly, they are often expressed in a subpopulation of endodermal cells and their inactivation usually inhibits the development of parts of the gut. In mouse, neither FoxA1- nor FoxA3-inactivated mutants exhibit any early phenotype (Kaestner et al., 1998). By contrast, FoxA2, which is expressed at the onset of gastrulation, is required for fore- and midgut formation (Ang et al., 1993; Dufort et al., 1998; Sasaki and Hogan, 1993; Weinstein et al., 1994).

In zebrafish, *Gata5*/*fau* is expressed before FoxA2, suggesting that it is upstream of this forkhead transcription factor, as also described in sea urchin, C. elegans and Drosophila (Azzaria et al., 1996; Casanova, 1990; Horner et al., 1998; Kalb et al., 1998; Mango et al., 1994; Weigel et al., 1989). Direct regulation by Tcfs and T-boxes have been characterized in sea urchin and Ciona but remain to be investigated in other species (Davidson et al., 2002; Di Gregorio et al., 2001). Furthermore, the presence of Smad2 binding elements in the *Xenopus Foxa2* promoter raises the possibility that it is a direct nodal target (Howell and Hill, 1997). Autoregulatory loops-positive or negative- have been demonstrated in different species. (Davidson et al., 2002; Di Gregorio et al., 2001).

FoxA targets have been studied comprehensively in *C. elegans* (Gaudet and Mango, 2002). Beyond compiling a list of targets, Mango and co-authors have shown that the late targets have lower affinity binding sites and thus are only induced once the levels of FoxA reach a critical threshold. Several studies have proposed that GATAs and FoxA together form a preinitiation complex that is required but not sufficient for endoderm gene transcription (Bossard and Zaret, 1998; Cirillo et al., 2002).

3.5. Sox and Mix, vertebrate players

Specific to vertebrates, other key components of the network downstream of Nodal comprise *Sox17*, *Mix*, and several related genes.

3.5.1. Sox17

Sox17 was first implicated in endoderm development in *Xenopus* and has also been extensively studied in Zebrafish where there are several members of the family (Alexander and Stainier, 1999; Hudson et al., 1997). In mouse, Sox17 is first expressed in visceral endoderm nearest to the ectoplacental cone at 6.0 dpc and progressively spreads to the entire extraembryonic VE. It is also expressed in definitive endoderm from 7.5–8.5 dpc (Kanai-Azuma et al., 2002). Mid- and hindgut expression persists until 8.5 dpc, whereas foregut expression decreases by 8 dpc. In *Sox17* knockout mice, definitive endoderm is depleted and visceral endoderm-like tissue replaces it in the most posterior and lateral

regions. Anterior endoderm is generated, but posterior and lateral endoderm down from the midgut level are reduced and later fail to expand (Kanai-Azuma et al., 2002). In contrast to *Foxa2*-knockout cells that can form hindgut but not fore- and midgut, *Sox17* mutant cells can contribute to some extent to the foregut but not mid- and hindgut (Dufort et al., 1998). Elevated levels of apoptosis in the foregut later lead to foregut reduction suggesting that Sox17 is also a maintenance factor for endoderm. Promoter studies in *Xenopus* have demonstrated that Sox17 is directly regulated by TGFβs (Vg1 or Nodal) and through a different promoter element by Sox17 itself and VegT (Howard et al., 2007). Mixer also participates in Sox17 regulation by stimulating an autoregulatory loop which also involves GATAs (Sinner et al., 2006). Sox17 directly activates the endodermal genes *HNF1β, FoxA1, FoxA2* and *Endodermin* in *Xenopus*, in part through synergistic interactions with β-catenin (Ahmed et al., 2004; Sinner et al., 2004). Other endodermal genes are exclusively under transcriptional control by Mixer or require synergy between Mixer and Sox17 (Sinner et al., 2006). In mice, Sox7 and Sox17 may be redundant in extraembryonic visceral endoderm.

3.5.2. Mix family

The Mix family encodes homeodomain proteins initially described in *Xenopus*. Mixer is predominantly expressed at the endoderm/mesoderm boundary, and is the only gene of the family that induces endoderm specifically (Henry and Melton, 1998; Kofron et al., 2004; Sinner et al., 2006). Mix1 and Bix1/Mix4 induce endoderm at high levels and can repress mesodermal genes like Xbra whereas at low level, they induce mesoderm (Henry and Melton, 1998; Latinkic and Smith, 1999; Latinkic et al., 1997; Lemaire et al., 1998; Tada et al., 1998).

Only one *Mix* gene has been found in Amniotes (Peale et al., 1998; Pearce and Evans, 1999; Robb et al., 2000; Stein et al., 1998). Mouse *Mixl1* is first detected in the visceral endoderm and later in nascent primitive streak, but not in the node or definitive endoderm. Mice lacking Mixl1 have reduced definitive endoderm and mid- hindgut lies at the level of the foregut (Hart et al., 2002; Tam et al., 2007). However, visceral endoderm is displaced normally to the periphery. Mixl1 mutant cells in chimeras contribute to all organs but the hindgut. Conversely, Mixl1 overexpression in frog injection assays can induce excess endoderm formation (Hart et al., 2002). *Nodal* expression is expanded in *Mixl1* mutants suggesting that a feedback loop regulates NODAL. SMAD2/4 dimers bind the activin-responsive element of the mix2 promoter (Howell et al., 1999). Mixer recruits SMAD2/4 to activin responsive elements of mesendodermal genes such as Gsc (Germain et al., 2000).

In addition to these genes, many genes have been described in endoderm and may be used as markers as compiled in Table 1.

4. Engineering endoderm

4.1. Generating endoderm from ES cells using Activin or Nodal

ES cells spontaneously form endoderm, including definitive endoderm, but in a small proportion (Itskovitz-Eldor et al., 2000). Early work aimed at generating clinically relevant endodermal derivatives from ES cells did not characterize intermediate steps in the differentiation and rather focused on the end products such as hepatocytes or pancreatic beta cells. Such attempts were characterized by either controversy in their reproducibility at generating functional differentiated cells (Hori et al., 2002; Lumelsky et al., 2001; Rajagopal et al., 2003) or their low efficiency (Hamazaki and Terada, 2003; Jones et al., 2002; Blyszczuk et al., 2003; Vincent et al., 2006; Yamada et al., 2002; Yamada et al., 2002). Based on the knowledge of endoderm development, strategies have more recently been devised to generate endoderm from mouse and human embryonic stem cells (ESCs). Developmental knowledge provided triggers and markers in this process. As in vivo, Nodal is necessary for endoderm induction from ES cells (Gadue et al., 2006; McLean et al., 2007). Due to the limited availability and price of biologically active Nodal protein (Tada et al., 2005), most efforts have made use of Activin as a surrogate for Nodal. Initial experiments by Kubo et al. (Kubo et al., 2004) and Yasunaga et al. (Yasunaga et al., 2005) on mouse ES cells (mESCs) and D'Amour et al. (D'Amour et al., 2005) on human ES cells (hESCs) have shown that endoderm is efficiently generated with both species in the presence of Activin and low serum. This protocol functions with mESCs aggregated into embryoid bodies (Kubo et al., 2004) as well as mESCs or hESCs cultured as a monolayer (D'Amour et al., 2005; Yasunaga et al., 2005). The efficiency of the protocol appears to vary largely depending on the cell lines used but has been successfully used in many laboratories (D'Amour et al., 2006). The low serum most likely limits phosphatidyl Inositol 3 kinase (PI3K) activity, a condition needed for definitive endoderm formation from ES cells (McLean et al., 2007). Nodal has also in some instances been provided by the use of MEF-conditioned medium (McLean et al., 2007).

Table 1. Selected endoderm-enriched genes Yellow: membrane proteins; green: membrane proteins used for cell sorting. (HSCs) Hematopoietic stem cells (OV) Otic vesicle (YS) Yolk Sac (NT) Neural tube

Gene name	ES	E7.5	E8.5 Fore	E8.5 Mid	E8.5 Hind	E9.5 Fore	E9.5 Mid	E9.5 hind	E10.5 and +	Extraemb. endoderm	Other tissues	References
General endoderm												
Sox17	−	+	±	+	+	?	?	?	?	VE	HSCs, endothelia	Kanai-Azuma et al., 2002; MGI
Foxa2	−	+	+	+	+	+	+	+	+	PE,PrE,AVE	notochord, floor plate	Kimura-Yoshida et al., 2007; Ang et al., 1993, emap
Claudin6	?		+	−	+	+	+	+	+	VE	epiblast, OV, mesonephos	Anderson et al., 2008; Sousa-Nunes et al., 2003; MGI
CXCR4	−	+	+	+	±	+	+	−	±	PE, YS	mesoderm, many after 8.5	McGrath et al., 1999; Yasunaga et al., 2005; MGI
Gata4,6	?		+	±	±	+	+	+	+	VE,PrE	heart	Hou emap(4); MGI(4)
Tcf2/HNF1b	−	−	?	+	+	post	+	+	+	VE	notochord	Sherwood et al., 2007; MGI
Tmprss2	−	+	?	+	+	?	+	+	+	VE	Prostate	Hou et al., 2007; Moore-Scott et al., 2007; Sherwood et al., 2007; MGI
Tacstd1 (EpCAM)	+	+	+	+	+	+	+	+	+	VE	Ectoderm, lower after 8.5	Sherwood et al., 2007; MGI
Embigin	−	+	+	+	−	?	±	±	−	VE,YS	midbrain, kidney	Huang et al., 1990; Sousa-Nunes et al., 2003; Fan et al., 1998; MGI
Hhex	−	+	+	−	−	+	−	−	+	PrE,PE,AVE	endothelia	Sherwood et al., 2007; MGI
Cer1	?	+	+	−	−	−	−	−	−	AVE	mesoderm	Sherwood et al., 2007; MGI
Dkk1	?	+	+	−	−	+	−	−	?	AVE	head mesoderm	Sherwood et al., 2007; MGI
AnxA4	+	?	−	+	+	−	+	+	?	VE	Notochord	Sherwood et al., 2007; MGI
Bnip1	+	?	±	±	±	−	+	+	?	VE	OV	Sherwood et al., 2007; MGI
Cacna1b	−	+	+			+			?	VE	heart	Sherwood et al., 2007; MGI
Clic6	−	?	?	+	+	±	+	+	+	VE	head mesenchyme	Sherwood et al., 2007; Hou et al., 2007, emap; MGI
Dsg2	+	+	+			+			?	VE	mesoderm	Sherwood et al., 2007; MGI
Krt7	−	+	+			+			?	VE, YS	?	Sherwood et al., 2007; MGI
Npnt	−	?	?	?	?	?	+	+	?	VE++	branchial arch 1	Sherwood et al., 2007; MGI
Rab15	−	?	?	?	?	?	±	±	?	VE++	OV	Sherwood et al., 2007; MGI
Rbm35a	+	±	±	±	?	+	±	±	?	?	OV	Sherwood et al., 2007; MGI
Ripk4	−	±	±	?	+	+	+	+	?	VE	OV	Sherwood et al., 2007; MGI
Sh3gl2	+	±	+			+			?	VE	?	Sherwood et al., 2007; MGI
St14	+	±	±			−	+	+	?	VE	branchial arch 1	Sherwood et al., 2007; MGI
Spink3	−	±	±	±	±	?	?	?	?	YS	ectoderm	Sherwood et al., 2007; Hou et al., 2007; MGI
Alpha Fetoprotein	−	−	+	+	+	liver	−	−	+	VE,YS	−	Dziadek and Adamson, 1978; MGI

Table 1. (Continued)

Gene name	ES	E7.5	E8.5 Fore	E8.5 Mid	E8.5 Hind	E9.5 Fore	E9.5 Mid	E9.5 hind	E10.5 and +	Extraemb. endoderm	Other tissues	References
Enriched definitive endoderm												
Foxa3		−	±	+	+	±	+	+	+	−	germ cells	Ang et al., 1993; Monaghan et al., 1993; MGI
Foxa1	−	+	±	+	+	±	+	+	+	−	forebrain	Monaghan et al., 1993, emap, MGI
PYY	?	−	+	−	−	+	−	−	+	−	brain	Hou et al., 2007; MGI
5730521E12Rik/ Nephrocan	−	+	−	+	+	−	+	−	+	−	kidney	Hou et al., 2007; Sherwood et al., 2007; MGI
NM_029639	?	+	−	−	+	+	+	+	+	−	Oral ectoderm	Moore-Scott et al., 2007
Klf5	?	+	−	+	+	−	+	+	+	−	Otic vesicle, somites, brain	Moore-Scott et al., 2007; MGI
Epha2	?	+	+	−	+	−	−	+	+	−	limb	Moore-Scott et al., 2007; MGI
Cdx 2	?	+	−	−	+	−	−	+	+	−	Trophectoderm NT, notochord	Hou et al., 2007, emap; MGI
Enriched in extraembryonic endoderm												
Ceacam1	+	−	−	−	−	−	−	−	−	VE++ (8.25)	?	Sherwood et al., 2007; MGI
Dpp4	−	−	−	++	−	−	++	−	?	VE++ (8.25)	Somites+/−	Sherwood et al., 2007; MGI
Icam	+	−	−	−	−	−	−	−	−	VE++ (8.25)	?	Sherwood et al., 2007; MGI
CD38	+	−	−	−	−	−	−	−	−	VE++ (8.25)	?	Sherwood et al., 2007
Timd2	+	−	−	−	−	−	−	−	−	VE++ (8.25)	?	Sherwood et al., 2007; MGI
DBA lectin	±	−	−	−	−	−	−	−	−	VE (8.25)	?	Sherwood et al., 2007
SSEA-4	−	−	−	−	−	−	−	−	−	VE (8.25)	?	Sherwood et al., 2007
Claudin9,habp2, spp2,ttr	?	?	±	±	±	?	?	?	?	YS	?	Hou et al., 2007
Amn	−	−	−	−	−	?	?	?	?	PrE,VE	?	Sherwood et al., 2007; Kalantry et al., 2001; MGI
HNF4alpha	−	−	−	−	−	−	−	−	liver	VE,YS		Sherwood et al., 2007; Vincent and Robertson, 2004; MGI
Cited1	?	−	−	−	−	−	−	−	−	VE	Heart, lateral mesoderm	Rodriguez et al., 2004; Sherwood et al., 2007, emap; MGI
Sox7	?	−	−	−	−	−	−	−	−	VE	endothelia	Kanai-Azuma et al., 2002; MGI
Tdh	?	−	?	?	?	?	?	?	?	YS	?	Hou et al., 2007; MGI
Lgals1	?	−	?	?	?	?	?	?	?	YS	?	Hou et al., 2007; MGI
Cubn	?	−	?	?	?	?	?	?	?	YS	?	Hou et al., 2007; MGI
Pla2g12b	?	−	?	?	?	?	?	?	?	YS	?	Hou et al., 2007; MGI
Apoc2	?	−	?	?	?	?	?	?	?	YS	?	Hou et al., 2007; MGI
Slc39a8	?	−	+	+	+	±	±	±	?	VE	endothelia	Moore-Scott et al., 2007; Sousa-Nunes et al., 2003; MGI
Angiomotin	?	+	?	?	?	?	?	?	?	VE	?	Moore-Scott et al., 2007; Shimono and Behringer, 2003; MGI
Dp111/Reep6	?	−	?	?	?	−	−	+	+	VE	brain	Moore-Scott et al., 2007; MGI

(Emap) Edinburgh mouse Atlas Project, (MGI) mouse genome Informatics. In addition to the references herein, markers in Zebrafish may be found in Brown et al. 2008.

As expected from the developmental response to Nodal morphogen the authors could demonstrate that 25–100 ng/ml activin lead to 50–60% endoderm whereas lower doses of activin (1–10 ng/ml) induced skeletal muscle markers (Gadue et al., 2006; Kubo et al., 2004). The duration of activin exposure was explored in less detail but Kubo et al. reported that hematopoietic progenitors emerged after 5 days of activin (3–100 ng/ml) treatment whereas 6 days of exposure were necessary for induction of the endodermal markers Sox17 and Hex. Shorter durations of high activin exposure (3 days) can nevertheless induce endoderm (Yasunaga et al., 2005), in particular when Wnts are added (D'Amour et al., 2006).

More recently the tetracycline-inducible system was used to drive expression of Nodal and was demonstrated to be more effective at inducing endoderm that adding exogenous Activin (Takenaga et al., 2007). This may be due to different intrinsic properties of Nodal and activin. Although they share the same receptors, their mechanism of action is somewhat different as for example their different requirement for Cripto. The differences observed may however be due to the method of delivery: endogenously produced Nodal may be differently post-translationnally processed, may traffic differently or may be expressed more evenly.

4.2. Molecular characterization of endoderm induced from ES cells

The expression of Foxa2 and Sox17 show that the endoderm induced is at least in majority definitive endoderm. This idea is also confirmed by the transient expression of primitive streak markers such as Brachyury, Goosecoid, Lhx1, MixL1, PDGFRα and Wnt3a (D'Amour et al., 2005; Gadue et al., 2006; Kubo et al., 2004; Yasunaga et al., 2005). ES cells have been used to decipher the hierarchy of protein activity downstream of activin, confirming that Eomesodermin is acting upstream of Mixl1 during endoderm differentiation (Izumi et al., 2007; Russ et al., 2000). Of particular interest, a recent study comparing two protocols of endoderm induction from ES cells reported a list of potential new endoderm markers using microarray RNA profiling of the genes enriched in both conditions (McLean et al., 2007). An earlier microarray study provided several additional validated markers including Cxcr4 that can be used to sort cells (Yasunaga et al., 2005). The eventual test for their endodermal nature will be transplantation assays and the proof that they can integrate to mouse or chick endoderm and further differentiate and contribute to endodermal organs.

4.3. Role of the Wnt pathway in endoderm induction from ES cells

Although the most ancient signaling pathway for endoderm induction has been marginally used for endoderm induction, Wnt pathway activity is necessary during endoderm induction from ES cells (D'Amour et al., 2006; Gadue et al., 2006; Lindsley et al., 2006). Wnt3 (10ng/ml or 100 ng/ml) on its own does not have the ability to induce endoderm but at least in some instances can potentiate Activin activity (D'Amour et al., 2006; Gadue et al., 2006). During development, many other genes described in the previous paragraphs are necessary to form endoderm but there is limited knowledge as to whether they may be sufficient to induce endoderm from ES cells. Forced expression of Sox17 was rtecently shown to promote endoderm differentiation from ES cells (Seguin et al., 2008).

4.4. ES cells as a tool to answer developmental and medical questions

Experiments with ES cells are helpful to answer questions that may be difficult to address in mice or human. The best example is the demonstration using ES cells of mouse mesendoderm progenitors. Clonal analysis showed that sorted Gsc+ single cells generated by activin treatment of ES cells could generate clones made of mesoderm only or mesoderm and endoderm (Tada et al., 2005). This experiment provides strong support in favor of mesendoderm progenitors and the lack of endoderm-only progenitors. ES cells may be very useful to study the antero-posterior commitment of endoderm cells. ES cells may also provide the number of cells needed to perform biochemistry or chromatin immunoprecipitations in endoderm-like cells. In the longer term, it would be interesting to have endodermal stem cells that can only give rise to endodermal lineages and be stably maintained as has been achieved for the ectoderm (Conti et al., 2005; Tada et al., 2005).

Lastly, human ES cells with mutated genes could also represent a wonderful tool to study endodermal organ disease.

5. Endoderm regionalization and morphogenesis

5.1. Markers and fate maps reveal progressive patterning of endoderm into organs

In chick and mouse, the cells recruited early in the primitive streak will form more anterior endoderm derivatives. The position of endoderm progenitors along the primitive streak reflects their later antero-posterior (AP) and

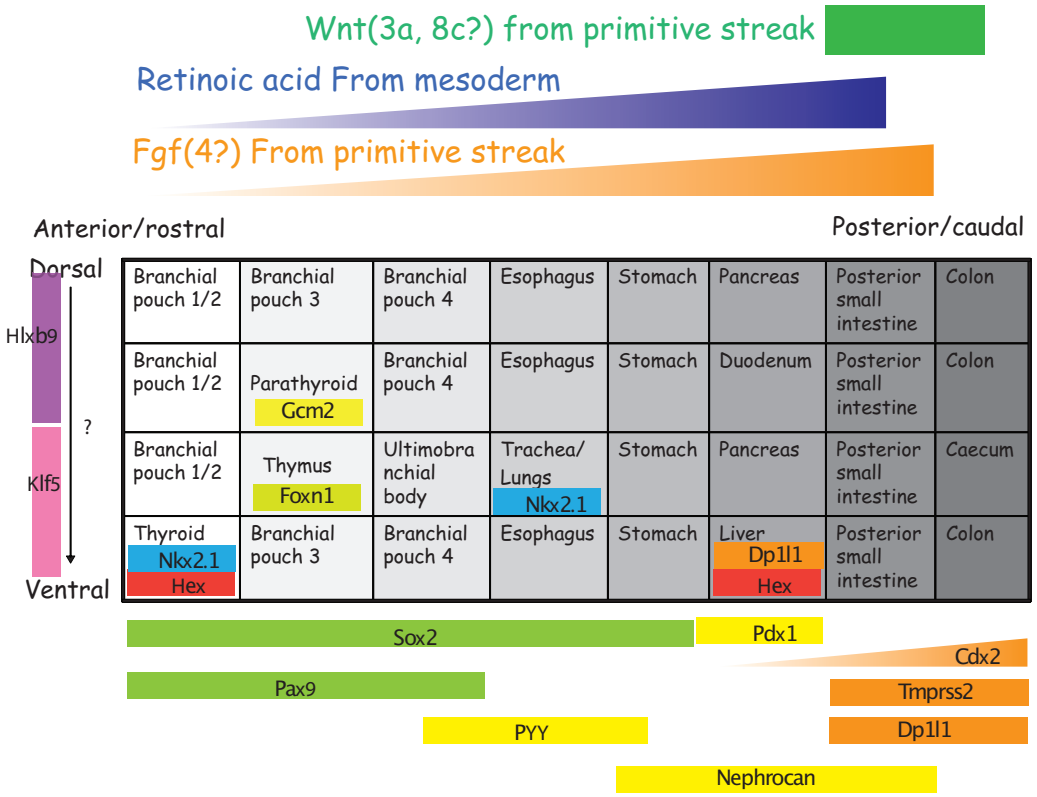

Figure 5. Origin of endoderm cells eventually populating different gut areas in chick and mouse. Yellow marks prospective dorsal foregut endoderm, green marks prospective ventral foregut endoderm, red marks prospective dorsal mid- hindgut endoderm and orange marks prospective ventral mid-hindgut endoderm. The panels on the left show cells in the streak just before migration. All other panels show their emergence in the endodermal layer. Ventral foregut endoderm progenitors shown in green have not been mapped in mouse and in the chick streak. Picture of early streak embryo courtesy of D. Mesnard. Regional markers of endoderm. Endoderm can be schematized as a 2-D plane with AP and DV axes. On this plane organ domains emerge at specific locations. Markers with relatively stable expression patterns between 8.5/9.5 and 10.5 dpc are presented with colored boxes. Endoderm is regionalized through the activity of secreted factors, which origin is indicated. Among those at least Fgfs and retinoic acid have a graded activity along the AP axis. Secreted factors patterning the DV axis are awaiting.

medio-lateral position (Franklin et al., 2008; Lawson et al., 1986; Tam et al., 2004). Subsequently, folding of the gut tube anteriorly brings the most anterior cells to the most ventral positions (Franklin et al., 2008).The endoderm at this stage appears to be roughly divided into anterior and posterior areas. *Cerberus 1 (m-Cer1/Cerr)1* (Belo et al., 1997; Biben et al., 1998; Shawlot et al., 1998), *Orthodenticle homologue 2 (Otx2)* (Ang et al., 1994), *Homeo box gene expressed in ES cells 1 (Hesx1)* (Thomas and Beddington, 1996) and *Hematopoietically expressed homeobox (Hex)* (Thomas et al., 1998) are restricted to anterior endoderm. Antero-posterior asymmetry of the endoderm at the same stages is also demonstrated by the specific ability of the anterior endoderm to induce heart differentiation in the mesoderm (Schultheiss et al., 1995). However, at this early stage association of the anterior endoderm half with posterior mesoderm can still induce posterior genes in endoderm and vice versa (Wells and Melton, 2000), suggesting that AP patterning of the endoderm is not yet determined. At somitic stages, the identity of more regions exhibiting different gene expression profiles is progressively specified (Grapin-Botton, 2005; Grapin-Botton and Melton, 2000).

5.2. Molecular mechanisms of endoderm patterning

The molecular mechanisms responsible for early patterning along the AP and medio-lateral/dorso-ventral axes in endoderm are beginning to emerge. They are schematized in Figure 5. Thus far, they appear to be very similar to the mechanisms of AP patterning of the neurectoderm, involving wnts, Fgf4 and retinoic acid. Retinoic acid was recently shown to control AP patterning in endoderm at the time of gastrulation in Xenopus, Zebrafish and Amphioxus (Chen et al., 2004; Escriva et al., 2002; Schubert et al., 2005; Stafford et al., 2004; Stafford and Prince, 2002; Stafford et al., 2006). This work suggests that increasing levels of retinoic acid activity gradually induces posterior organs. In mouse retinoic acid is required to form the pancreas and pattern branchial arch endoderm (Huang et al., 2002; Huang et al., 1998; Martin et al., 2005; Matt et al., 2003; Molotkov et al., 2005). However, it is unclear whether graded activity orchestrates the relative position of organs along the entire AP axis in this layer. Retinoic acid is produced

by the mesoderm, a tissue that sends patterning signals to endoderm (Kumar et al., 2003; Pan et al., 2007; Wells and Melton, 2000). Fgfs are also necessary for endoderm patterning from gastrulation to somitogenesis (Dessimoz et al., 2006; Serls et al., 2005; Wells and Melton, 2000). Exposing endoderm to FGF4, a node-derived factor, shifts posterior endoderm markers anteriorly and represses anterior markers at gastrulation. After gastrulation the patterning role of FGF4 becomes restricted to the mid- and hindgut, where increasing levels of signalling progressively induce more posterior fates in endodermal cells (Dessimoz et al., 2006; Wells and Melton, 2000). Although these pathways appear to pattern endoderm at least in part through direct signaling to this layer, more work is needed to elucidate how they cooperate (Dessimoz and Grapin-Botton, 2006; Huang et al., 2002; Pan et al., 2007; Stafford et al., 2006). Recent experiments in Xenopus show that endoderm is also patterned by the Wnt pathway: the foregut forms in the absence of Wnt activity whereas Wnt signalling is necessary to form the intestine (McLin et al., 2007). The lack of anterior Wnt activity is permitted by the expression of several Wnt inhibitors (Cerberus, dickkopf, Frzb) in anterior endoderm (Belo et al., 1997; Lewis et al., 2008; Mukhopadhyay et al., 2001; Pfeffer et al., 1997). The role of this pathway in endoderm development is likely to be conserved in rodents as mice deficient for Tcf1 and Tcf4 exhibit posterior endoderm defects (Gregorieff et al., 2004).

5.3. Local signals lead to organ formation

There is evidence that in addition to the signals that lead to the regionalization of endoderm along the AP axis, other signals are necessary locally for the induction of organ primordia. These signals are discussed in more details in the chapters pertaining to specific endoderm organs. One such signal is Fgf2 secreted by the cardiac primordium which is necessary to liver and lung development (Deutsch et al., 2001; Gualdi et al., 1996; Jung et al., 1999; Serls et al., 2005). Similarly, BMP4 from the septum transversum is necessary for liver induction whereas the pancreas appears to form in the absence of BMPs (Rossi et al., 2001; Spagnoli and Brivanlou, 2008). In contrast to most of the digestive tract epithelium, the pancreas can only develop in an area free of Shh (Apelqvist et al., 1997). Lastly, Fgf10 is necessary for the development of several organs budding off the digestive tract such as the pancreas, caecum, lungs and stomach glands (Bhushan et al., 2001; Burns et al., 2004; Nyeng et al., 2007; Sekine et al., 1999).

5.4. Patterning endodermalized ES cells

In spite of our limited knowledge of endoderm patterning, the exploitation of such information to generate organ-restricted progenitors is promising. Exposure to FGF and BMP after endoderm induction by activin enriched in liver progenitors (Gouon-Evans et al., 2006). The role of retinoic acid, Fgf10 and the absence of Shh in pancreas development were exploited to enrich endodermalized ES cells in pancreas progenitors (D'Amour et al., 2006; Kroon et al., 2008). Since Nodal and Wnts used to induce endoderm from ES cells also play a role in AP patterning, a careful investigation of the regional endoderm markers obtained after different amounts and duration of exposure to these morphogens would be interesting. Further, ES cells could be very valuable to investigate that are difficult to address in vivo such as the possible graded activities of Nodal, Wnt, RA and Fgf signaling pathways in endoderm patterning.

6. Differentiation of extraembryonic endoderm lineages

In mouse, primitive endoderm (PrE) segregates from the inner cell mass (ICM) at the blastocyst stage as a squamous epithelium and covers the outside of the embryo (Weber et al., 1999; see Figure 2). Whereas some PrE cells remain attached to the basement membrane of the ICM and differentiate into cuboidal visceral endoderm (VE), others undergo an epithelial-mesenchymal transition to become parietal endoderm (PE). PE cells migrate along the basement membrane of trophectoderm (TE) cells which gives rise to Reichert's membrane of the parietal yolk sac. VE forms the epithelial lining of the yolk sac and some cells contribute to the definitive gut tube at least until 9.0dpc (Kadokawa et al., 1987; Kwon et al., 2008). Until placentation, PE and VE lineages together are responsible for nutrient and waste exchange between maternal tissue and the foetus. VE can contribute in a minor way to embryonic gut in the fore- and hindgut (Tam and Beddington, 1992). What we know of the differentiation of these 3 lineages arises from a cross-talk between in vivo experiments and experiments with ES cells. ES cells are thus already an experimental model of embryo development. Markers of extraembryonic endoderm lineages, some of which are shared with definitive endoderm are indicated in Table 1.

6.1. Primitive endoderm (PrE)

Before the segregation of epiblast and PrE, the inner cell mass (ICM) is a mosaic of cells expressing markers of one or the other lineage (Gerbe et al., 2008; Plusa et al., 2008). In ES cell lines and in vivo, Pou5f1 (Oct3/4) is required to maintain pluripotency and to prevent differentiation into TE cells (Nichols et al., 1998; Niwa et al., 2000). On the

other hand, using an inducible Pou5f1 transgene, less than twofold increase in the expression level of Pou5f1 in ES cells is sufficient to induce markers of PrE and mesoderm differentiation (Niwa et al., 2000). Peak levels of Pou5f1 expression thus are likely to also specify PrE in the blastocyst. In normal ES cell cultures, differentiation is inhibited owing to the presence of LIF. However, if ES cells are aggregated to form embryoid bodies (EB), LIF can no longer prevent PrE differentiation and the segregation of these cells at surface of these aggregates (Murray and Edgar, 2001; Shen and Leder, 1992). Zebrafish MZ-Spg/Pou2/0ct4 mutants lack endodermal markers such as Cas/Sox32 and Sox17 but a similar activity in Amniotes may be hidden by early lethality (Lunde et al., 2004; Reim et al., 2004).

Communication between ICM cells is needed to segregate PrE from epiblast. Mice lacking either *Fgf4*, *Fgf receptor2* (*Fgfr2*) or their downstream effector Grb2 do not form PrE (Arman et al., 1998; Feldman et al., 1995; Wilder et al., 1997; Chazaud et al., 2006; Cheng et al., 1998). In embryoid bodies, forced expression of a dominant-negative form of the Fgf receptor prevents formation of PrE, and overexpression of *Gata6* and *Gata4* rescues this phenotype (Li et al., 2004; Li et al., 2001). Gata factors are thus likely to control PrE formation downstream of Fgf signaling, although single knockouts for these genes do not elicit such early phenotypes, most likely due to redundancy (Hamazaki et al., 2006; Koutsourakis et al., 1999; Morrisey et al., 1998). The Fgf signaling pathway represses Nanog expression and Nanog represses Gata6 (Hamazaki and Terada, 2003). It is however unclear how an apparently random subset of ICM cells activate the FGF pathway. Several pieces of evidence suggest that Gatas control the sorting of PrE and epiblast cells (reviewed in (Yamanaka et al., 2006). Apoptosis may lead to the elimination of inappropriately sorted cells (Plusa et al., 2008). Genes enriched in PrE have been identified by gene expression arrays (Gerbe et al., 2008). Extraembryonic stem cells (XEN) have been isolated from PrE and contribute to their lineage of origin in chimeras (Kunath et al., 2005). Overexpression of GATA factors in ES cells leads to cells molecularly very similar to XEN cells and both contribute preferentially to parietal rather than visceral endoderm in chimeras (Shimosato et al., 2007). Subsequent withdrawal of GATAs however leads to their differentiation into cells endowed with visceral endoderm characters (Shimosato et al., 2007). Although XEN cells require mouse embryonic fibroblasts (MEFs) for their growth, GATA-induced ES cells do not, suggesting that MEF-derived factor(s) serve to maintain Gata expression.

6.2. Parietal endoderm (PE)

How PrE cells choose between PE and VE fates is poorly understood. Clonal analysis of cells from genetically marked E3.5 and E6.5–7.5 donor embryos revealed that PrE and VE both give rise to PE when transplanted into host blastocysts (Gardner, 1982). This suggests that PrE descendants adopt PE fate because of environmental cues, rather than owing to a lack of competence to express the characteristics of VE.

Terminally differentiated PE cells have been derived *ex vivo* from ES cells upon forced expression of GATA4 or GATA6 (Fujikura et al., 2002). The signals governing the differentiation of extraembryonic endoderm have been studied best in F9 cells. Upon treatment with retinoic acid, this embryonal carcinoma cell line differentiates to become PrE and VE (Strickland and Mahdavi, 1978; Strickland et al., 1980). The transcription factor Sox7 is needed downstream of RA for the induction of GATA4 and GATA6, and subsequent parietal endoderm differentiation (Futaki et al., 2004). Although Sox17 is not required for PE and VE formation from PrE in vivo recent work with ES cells shows a requirement in vitro (Shimoda et al., 2007). The effect of RA on F9 cells appears to be mediated by activated Ras (Verheijen et al., 1999). Parathyroid hormone-related peptide (PTHrP) produced by trophoblast cells and the deciduum immediately adjacent to the implantation site or induces an epithelial to mesenchymal transition (EMT) in PrE cells and their conversion into a PE-like cells (Behrendtsen et al., 1995; Chan et al., 1990; Karperien et al., 1996; Smyth et al., 1999; Strickland et al., 1980; Veltmaat et al., 2000). cAMP, the intracellular mediator of PTHrP triggers the same effect.

6.3. visceral endoderm (VE)

Differentiation and survival of VE depends on a number of transcription factors, including the orphan nuclear receptor HNF4 (Chen et al., 1994; Duncan et al., 1997). HNF4 is expressed in PrE as early as day E4.5, but after E5.25 becomes restricted to the visceral yolk sac endoderm (Duncan et al., 1994; Mesnard et al., 2006). Signals upstream of HNF4 include BMP2/4 (Coucouvanis and Martin, 1999), and the activin receptor Alk2 (Sirard et al., 1998) which is essential in extraembryonic lineages (Gu et al., 1999). Also the homeodomain protein HNF1ß and GATA6 are required to induce HNF4 expression in the VE (Barbacci et al., 1999; Coffinier et al., 1999; Morrisey et al., 1998), suggesting that both of these transcription factors may act within or in parallel to the BMP pathway. HNF1ß (Tcf2) also stimulates expression of HNF1α (Tcf1) and Foxa2 (Barbacci et al., 1999). GATA6 is responsible for activating expression of GATA4 (Morrisey et al., 1998), which in turn acts in the VE lineage to enable ventral closure of the primitive gut tube (Molkentin et al., 1997; Narita et al., 1997). Toghether, these observations indicate that differentiation of the VE

lineage is brought about by the concerted action of a cascade of transcription factors which later also regulate gene expression in the definitive endoderm and its various derivatives.

The rules as to how specific combinations of these and other transciption factors might pattern the VE remain poorly understood. Initially, VE cells form a columnar epithelium which is subsequently patterned along the proximal distal axis of the conceptus by inductive interactions with adjacent ectodermal cells (Brennan et al., 2001; Dziadek, 1978; Gardner, 1982). Single-cell labelling at E5.5 shows that cells can contribute to embryonic and extraembryonic visceral endoderm (Perea-Gomez et al., 2007). The AVE is formed from a PrE population of Cerl-expressing cells and cells that acquire Cer1 expression later (Torres-Padilla et al., 2007). Thus, expression of HNF1ß and TTF (Transthyretin) is confined to the extraembryonic region, whereas VE cells overlying the egg cylinder express α-fetoprotein (Dziadek and Adamson, 1978) and Ihh (Becker et al., 1997; Belaoussoff et al., 1998) and adopt a squamous morphology. Recent experiments have shown that Nodal is expressed in the PrE. Nodal signaling is essential to downregulate a subset of PrE markers and thus induce embryonic visceral endoderm (Mesnard et al., 2006). In the embryonic region, patterning of the VE further becomes evident with the expression of Wnt3 in the proximal-posterior region. By contrast, cells differentiating at the distal tip express elevated levels of Otx2, and in response move to the prospective anterior pole to become anterior visceral endoderm (AVE; Kimura et al., 2000). Foxa2 binds *Otx2* promoter and is essential for its expression in AVE (Kimura-Yoshida et al., 2007). The specific gene expression pattern of the distal tip is restricted by signalling from the extraembryonic ectoderm as well as nodal signalling (Mesnard et al., 2006; Rodriguez et al., 2005). The AVE also expresses a number of other specific markers, including Lefty-1 (Ebaf) and Cerberus-like. These secreted proteins function redundantly as negative feedback inhibitors in the Nodal pathway and thereby confine primitive streak formation to the posterior epiblast (Perea-Gomez et al., 2002).

7. Concluding remarks

Although our understanding of endoderm development is less extensive than that of ectoderm and mesoderm, it has been successfully exploited to generate endodermal cells from ES cells with high efficiency but not yet from induced Pluripotent Stem cells (iPS cells). This knowledge has also been helpful with regards to quality control by providing a set of markers that collectively define endoderm identity. This protocol is expensive and labour intensive. It would therefore be of great interest to develop endodermal stem cells endowed with the ability to self-renew and differentiate into all endodermal organs. Promising results in this direction have recently been published. Sorted Cxcr4/Hex double positive cells generated after endoderm induction from mES cells are likely to represent an anterior definitive endoderm population that can be stably propagated and further differentiated into liver and pancreatic cell types (Morrison et al., 2008). Forced expression of Sox17 in hEScells leads to the differentiation of mesendodermal cells which can also be propagated and further differentiated into liver and pancreatic cell types (Seguin et al., 2008). A major gap remains to be filled to understand how different organ primordia are induced from endoderm and use this strategy on endoderm cells in vitro. These recent advances in endoderm generation in vitro will most likely allow the development of new strategies to address questions that are difficult to address in vivo such as those requiring large numbers of cells or live monitoring of cells.

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