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Review

Distinct roles for visceral endoderm during embryonic mouse development

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Introduction

The murine visceral endoderm is an extraembryonic cell layer that appears prior to gastrulation and performs critical functions during embryogenesis. The traditional role ascribed to this cell layer entails nutrient uptake and transport. Besides synthesizing a number of specialized proteins that facilitate endocytosis, digestion, and secretion of nutrients, the visceral endoderm coordinates blood cell differentiation and vessel formation in the adjoining extraembryonic mesoderm, thereby ensuring efficient exchange of nutrients and gases between the mother and embryo. Recent studies suggest that in addition the anterior visceral endoderm (AVE) plays an active role in guiding early development of the embryo. Prior to being displaced by migrating definitive endoderm, AVE expresses gene products that specify cell fate and initiate axial patterning within the embryo. Here we highlight investigations that demonstrate the dual role for visceral endoderm during embryogenesis. We focus on studies showing that disruption of visceral endoderm function through gene targeting or misexpression affects development of the embryo proper.

Origin of the visceral endoderm

The visceral endoderm is a derivative of the primitive endoderm, or hypoblast, a transient cell layer that appears along the blastocoelic surface between days 4 and 5 of development (Gardner, 1983; Rossant, 1986; Hogan *et al.*, 1994). The primitive endoderm gives rise to two extraembryonic cell types, parietal and visceral endoderm (Fig. 1) (Casanova and Grabel, 1988; Hogan and Tilly, 1981; Hogan *et al.*, 1994; Boucher and Pedersen,

Abbreviations used in this paper: ActRIB, activin receptor IB; AFP, alpha-fetoprotein; AIP, anterior intestinal portal; A-P, anterio-posterior; ANB, anterior neural boundary; AVE, anterior visceral endoderm; BMP, bone morphogenetic protein; BMPR, bone morphogenetic protein receptor; CNS, central nervous system; CR-1, cripto-1; CRABP, cytoplasmic retinoic acid binding protein; CRBP, cytosolic retinoid binding protein; dbcAMP, dibutryl cyclic-AMP; D-V, dorso-ventral; FGF, fibroblast growth factor; HD, Huntington disease; HNF-3β, hepatocyte nuclear factor-3β; HNF-4, hepatocyte nuclear factor-4; p.c., post-coitum; RA, retinoic acid; RBP, serum retinol binding protein; Shh, sonic hedgehog; TGF-β, transforming growth factor-β.

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1996). The parietal endoderm, a single layer of cells that develops in association with trophectoderm, synthesizes large amounts of type IV collagen and laminins. These extracellular matrix proteins are assembled into Reichert's membrane, a specialized basement membrane that surrounds the embryo and passively filters nutrients (Gardner, 1983; Freeman, 1990). Visceral endoderm cells develop in association with the epiblast, or inner cell mass, and are morphologically and functionally similar to gut endoderm. Visceral endoderm cells have microvilli and contain numerous phagocytic and pinocytic vesicles (Fig. 2). These features allow efficient absorption and digestion of maternal nutrients. Visceral endoderm cells also synthesize and secrete proteins involved in nutrient transport, such as transferrin and apolipoproteins (Gardner, 1983; Freeman, 1990; Cross *et al.*, 1994). While parietal endoderm cells are terminally differentiated, visceral endoderm cells retain the ability to differentiate into parietal endoderm (Hogan and Tilly, 1981; Gardner, 1983).

At the egg cylinder stage of development, a layer of visceral endoderm covers the epiblast and the extraembryonic ectoderm, but cells comprising this endoderm layer are not uniform in appearance or function. Visceral endoderm cells overlying the lower or distal pole of the egg cylinder have a squamous morphology, while more proximal visceral endoderm cells are cuboidal (Gardner, 1983). These segmental differences in morphology are accompanied by regional differences in gene expression, as illustrated by the marker α-fetoprotein (AFP). Until 7.5 days postcoitum (p.c.), AFP is expressed only in more distal visceral endoderm covering the epiblast, and transplantation experiments demonstrate that AFP expression within visceral endoderm is actively inhibited by the extraembryonic ectoderm (Dziadek, 1978; Dziadek and Adamson, 1978; Dziadek and Andrews, 1983). Therefore, regional differences in gene expression may reflect interactions between visceral endoderm and underlying ectoderm.

Migration and functional differentiation of visceral endoderm cells

Around the time of gastrulation, visceral endoderm cells overlying the epiblast are gradually displaced by migrating definitive endoderm cells that originate from the embryonic ectoderm. Transplantation and dye injection experiments have demonstrated that definitive endoderm precursors ingress through the anterior end of the primitive streak and are incorporated into the midline endoderm (Lawson *et al.*, 1986; Lawson and Pedersen, 1987; Winkel and Pedersen, 1988; Tam *et al.,* 1993). Along with definitive endoderm precursors, the anterior primitive streak contains progenitors of the notochord and paraxial mesoderm. A small number of definitive endoderm cells may also migrate to the midline by direct delamination from the embryonic ectoderm (Tam *et al.*, 1993). Continued recruitment of embryonic cells to the definitive endoderm eventually excludes the vast majority of primitive endoderm derived cells from the invaginating foregut endoderm and the remainder of the embryo proper (Tam and Behringer, 1997; Thomas *et al.*, 1998).

In addition to being passively displaced by the expanding definitive endoderm, visceral endoderm cells overlying the epiblast undergo active migration. Beginning at 5.5 days p.c., there is polarized movement of visceral endoderm cells from the distal tip of the egg cylinder to the region overlying the future anterior aspect of the embryo (Thomas *et al.*, 1998). The mechanisms accounting for this movement are not known, but differential growth rates have been proposed as a possible explanation (Thomas *et al.*, 1998). The migration of visceral endoderm cells is accompanied by asymmetries in gene expression (see below), and emerging evidence suggests that these events provide important developmental cues for axial patterning.

Role of the visceral endoderm in nutrient uptake and delivery

The yolk sac is the final destination of migrating or displaced visceral endoderm cells. The yolk sac begins to form during gastrulation, as the proximal visceral endoderm becomes underlined by extraembryonic mesodermal cells (Figs. 1B, 3). These cell layers, together with the trophectoderm and parietal endoderm, functionally comprise an "early placenta", which is responsible for nutrient and waste exchange between days 5 and 10 of gestation (Cross *et al.*, 1994). The visceral yolk sac expands over the ensuing days of development, and blood islands, structures consisting of hematopoietic progenitors surrounded by a loose network of endothelial cells, appear (Fig. 4) (Dzierzak and Medvinski, 1995; Zon, 1995). Endothelial cell precursors associated with blood islands differentiate and coalesce to form a primitive circulation bed, which later becomes connected to the embryo via the vitelline vessels (Mustonen and Alitalo, 1995) .

The visceral endoderm overlying the extraembryonic mesoderm influences the differentiation and development of blood islands and vessels (Boucher and Pedersen, 1996). Classical studies with chick embryo explants demonstrated that removal of the endoderm layer disrupts erythropoiesis and blood island formation (Wilt, 1965; Milura and Wilt, 1969). Studies of hematopoiesis in early mouse embryos suggest that visceral endoderm plays an active role in induction of blood precursors (Belaoussoff *et al.*, 1998). Prestreak or early streak stage (6-6.5 days p.c.) embryonic ectoderm stripped of visceral endoderm is unable to give rise to primitive erythroblasts or express embryonic globin genes in culture, whereas recombination of embryonic ectoderm explants with visceral endoderm allows primitive hematopoiesis to proceed (Belaoussoff *et al.*, 1998). Intriguingly, these explant studies show that early gastrulation stage primitive endoderm can respecify anterior ectoderm, a lineage normally fated to become neuroectoderm, to develop into posterior cell lineages such as hematopoietic and angiogenic cells (Belaoussoff *et al.*, 1998). This process appears to be mediated by a diffusible factor secreted by posterior visceral endoderm (Belaoussoff *et al.*, 1998). Visceral endoderm also appears to influence the organization of the vascular network within the mesodermal layer of the yolk sac (Palis *et al.*, 1995; Bielinska *et al.*, 1996).

The importance of the visceral yolk sac in nutrient delivery has been demonstrated in a variety of experiments. Disruption of yolk sac endoderm function by injection of certain chemicals (e.g., trypan blue) or antibodies against yolk sac endoderm is teratogenic in rodents (Brent *et al.*, 1990; Jollie, 1990; Lloyd, 1990). Targeted deletion of genes encoding proteins critical for nutrient transport by the visceral endoderm, such as apolipoprotein B (Farese *et al.*, 1995) results in embryonic lethality by day 10 of development (Copp, 1995). Similarly, mutations affecting the

extraembryonic mesoderm and causing disruption of the blood cell or vessel formation, are lethal or result in severe defects in embryo development (Millauer *et al.,* 1993; Dickson *et al.,* 1995; Fong *et al.,* 1995; Healy *et al.,* 1995; Mustonen and Alitalo, 1995; Shalaby *et al.,* 1995; Winnier *et al.,* 1995; Boucher and Pedersen, 1996; Carmeliet *et al.,* 1996).

A transition in the nutrient delivery system of the embryo occurs when the allantois begins to fuse with the chorion at 9-10 days of development (Cross *et al*., 1994). Fetal vessels derived from the allantois interdigitate with maternal blood sinuses, resulting in a network of vascular cells that comprise the chorioallantoic or "mature" placenta. After 10 days p.c., the chorioallantois replaces the yolk sac as the primary means of nutrient, gas, and waste exchange.

Even after formation of the chorioallantoic placenta, the visceral yolk sac continues to play an indispensable role in development, as illustrated by studies on vitamin A and its derivatives, including retinoic acid (RA). Vitamin A deficiency causes distinctive developmental anomalies, which are recapitulated in mutant mice lacking both RA receptors (RARs) and retinoid X receptors (RXRs) (Gudas 1994; Kastner *et al.,* 1995, 1997a; Means and Gudas, 1995; Krezel *et al.,* 1996; Ghysenlick *et al.,* 1997; Subbarayan *et al.,* 1997). Several lines of evidence suggest that embryos initially accumulate retinoids in visceral endoderm of the yolk sac (Gudas, 1994; Means and Gudas, 1995; Johansson *et al*., 1997). Extraembryonic visceral endoderm cells synthesize extra- and intracellular RA-binding proteins, including serum retinol binding protein (RBP) and its receptors, as well as cytoplasmic RA-binding proteins (CRABPs) and retinoid binding proteins (CRBP) (Soprano *et al.,* 1986; Johansson *et al.,* 1997). In contrast, the chorioallantoic placenta does not express RBP, suggesting that the visceral endoderm of the yolk sac mediates retinol transfer to the embryo/fetus throughout gestation (Johansson *et al*., 1997).

Recent studies have shown that inhibition of visceral endoderm RBP synthesis by injection of antisense oligonucleotides into the yolk sac cavity produces spatiotemporal developmental anomalies (Bavik *et al*., 1996). Early introduction of RBP antisense oligonucleotides at 7.5 days p.c. causes a reduction in vitelline vessel formation. Injection at later stages results in neural tube defects, eye malformations, and decreased *sonic hedgehog (Shh)* expression. These effects can be reversed by co-injection of RA, emphasizing the importance of unperturbed retinoid delivery for normal organogenesis.

The differentiation of visceral endoderm itself may be influenced by RA. F9 embryonal carcinoma cells can be induced to form visceral or parietal endoderm by treatment with RA or RA/dibutryl-cAMP (dbcAMP), respectively (Wang *et al.,* 1985; Darrow *et al.,* 1990; Gudas, 1990, 1994; Wang and Gudas, 1990). Numerous studies have documented that this *in vitro* process requires expression of RARs, RXRs, CRABPs, and other genes that are either directly or indirectly induced by RA (Boylan *et al*., 1995; Chiba *et al*., 1997; Kastner *et al*., 1997b; Sapin *et al*., 1997; Taneja *et al*., 1997). Among such inducible genes are homeobox factors (Boylan *et al*., 1993, 1995; Becker *et al*., 1997), steroid hormone superfamily members like *Hnf4* (Duncan *et al*., 1997), the zinc finger transcription factors *Gata4* and *Gata6* (Soudais *et al*., 1995; Bielinska and Wilson, 1997), growth and differentiation factors such as *Indian hedgehog* (Becker *et al*., 1997), and

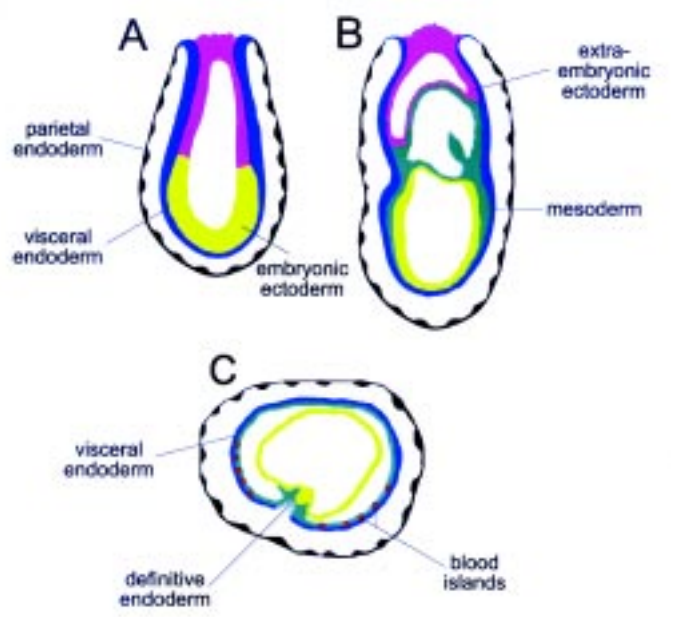

Fig. 1. Extraembryonic and embryonic layers in the early mouse embryo. (A) *Longitudinal section through a 6 day p.c. mouse embryo. The visceral endoderm (dark blue) covers the embryonic ectoderm, or epiblast (yellow), and extraembryonic ectoderm (purple). The parietal endoderm (black) envelops the embryo.* **(B)** *Longitudinal section through a 7.5 day p.c. mouse embryo. The mesoderm (green) is now evident.* **(C)** *Transverse section through a 7.7 day p.c. mouse embryo. The definitive endoderm (light blue) is marked. Blood islands (red) are evident at the junction between the visceral endoderm and extraembryonic mesoderm. Adapted from Kaufman (1992).*

extracellular matrix components and enzymes (Wang *et al.,* 1985; Gudas, 1990; Wang and Gudas, 1990; Sasaki *et al.*, 1991; Boylan *et al.,* 1993). However, it remains uncertain whether RA is necessary for *in vivo* differentiation of visceral endoderm. While disruption of RARα and RARγ results in specific changes in inducible gene expression, it does not block F9 cell differentiation (Boylan *et al*., 1995). None of the single or double mutants of RARs shows any obvious defect in visceral endoderm function or abnormalities in the early stages of embryonic development (Means and Gudas, 1995; Kastner *et al.,* 1997a,b). Furthermore, mice bearing a heat shock protein/β-galactosidase *(hsp/lacZ)* transgene containing a *RARβ*–derived RA response element exhibit reporter gene expression only after formation of the head folds (Rossant *et al*., 1991). While the RA inactivating enzyme cytochrome P450 is expressed in extracellular endoderm as early as 6 days p.c. (Fujii *et al*., 1997), visceral endoderm does not express enzymes involved in RA synthesis (Ang S.L. *et al*., 1996; Ang and Duester, 1997). Hence, *in vivo* there may be little role for RA in induction or differentiation of visceral endoderm prior to or during early stages of gastrulation.

Visceral endoderm and cavitation

Visceral endoderm covering the egg cylinder stage embryo plays a role in formation of the proamniotic cavity. The process of cavitation requires the presence of visceral endoderm and signals generated in the ectoderm provided by members of the Bone

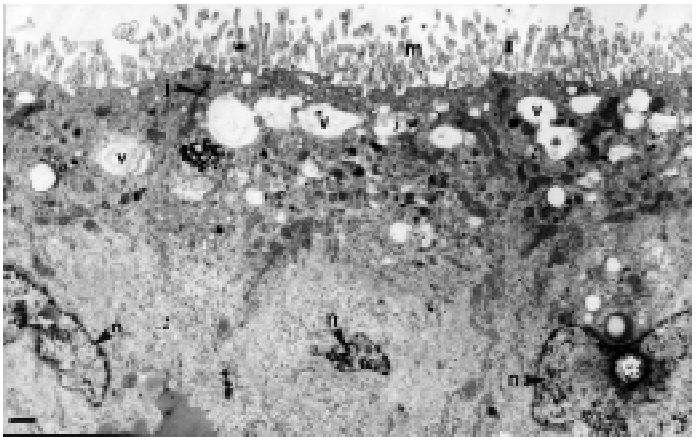

Fig. 2. Electron microscopy of columnar visceral endoderm cells. *Note the presence of microvilli, tight junctions, numerous phagocytic vesicles. Abbreviations: j, tight junction; m, microvilli; n, nucleus; v, phagocytic vesicle/ vacuole. Bar, 1 nm.*

Morphogenetic Protein (BMP) family (Coucouvanis and Martin, 1995, 1999). BMPs were originally identified in regenerating bone, but have since been shown to play a role in many developmental processes (Hogan, 1996). These proteins belong to the transforming growth factor-β (TGF-β) superfamily of signaling molecules. Once secreted, growth factors reside in the extracellular space, often in latent forms, which are proteolytically processed to active forms (Hogan, 1996). BMP signaling occurs via binding to BMP Receptor Type II (BMPR-II), which heterodimerizes with BMPR-I (Hogan, 1996; Massagué and Weis-Garcia, 1996). Homozygous null mouse mutants have been characterized for BMP-4 (Winnier *et al.*, 1995), BMPR-I (Mishina *et al.*, 1995), and BMP-2 (Zhang and Bradley, 1996). In each case, visceral endoderm morphology is affected, and the first two of these mutants exhibit early embryonic lethality with blocks in mesoderm formation and reduced ectoderm proliferation. In addition, it has been shown that BMP-4 signaling induces apoptosis in chick (Graham *et al.*, 1994; Ganan *et al.,* 1996) and in murine P19 cells (Glozak and Rogers, 1996).

BMP-4 is expressed in ectodermal cells prior to cavitation and its ectodermal expression ceases first in the embryonic and later in the extraembryonic ectoderm after the start of cavitation in both regions (Coucovanis and Martin, 1999). BMP-4 is not expressed in visceral endoderm, but there is weak expression of BMP-2 between 5.0 and 6.5 days p.c. Comparison of two embryonal carcinoma (EC) cell lines, one of which becomes covered with visceral endoderm and cavitates during differentiation into embryoid bodies (PSA1), and the other which becomes covered by a sparse single endoderm PE-like cell layer and does not cavitate (S2), has facilitated the study of the role of BMP-4 in the induction of cavitation (Coucovanis and Martin, 1999). Expression of a dominant negative BMPRIb in PSA1 cells prevented cavitation in embryoid bodies and reduced expression of visceral endoderm differentiation markers. In S2 cell derived embryoid bodies application of BMP-4, which can be substituted by BMP-2 or BMP-7, results in morphologically recognizable visceral endoderm layer, and in formation of small peripheral cavities containing apoptotic cells. BMP-4 emerges therefore as a signaling molecule involved in both apoptotic events leading to cavitation in the embryo and differentiation of visceral endoderm. During these early stages intersignaling between visceral endoderm and underlying epiblast may be crucial for executing early developmental cues.

Visceral endoderm as an anterior organizer

Early studies by Spemann and Mangold showed that in amphibian embryos the dorsal blastopore lip, a region which was subsequently termed the Spemann organizer, could induce a complete second axis when grafted in the ventral side of an embryo (Spemann and Mangold, 1924). Studies of body axis formation led to discovery of similar inducing centers in other vertebrates, which were called Hensen's node in chick (Waddington, 1933), the embryonic shield in zebrafish (Ho, 1992), and the node in mouse (Beddington, 1994). While grafts of the early Spemann organizer can induce a complete, head-containing secondary axis, grafts of late organizer tissue give rise to the trunk region without a head (Spemann, 1931). Node transplantation in the mouse embryo results in the formation of incomplete axis without anterior structures (Beddington, 1994), suggesting that the inducing activities for anterior and posterior identity reside in the different centers. Until recently, anterior patterning of the developing central nervous system in the mouse had been attributed solely to the influence of axial mesoderm or definitive endoderm derived from the node during gastrulation. Over the past few years it has become apparent that murine AVE, which overlies the future head and anterior embryonic region, plays an active role in the specification of the anterio-posterior (A-P) axis as well as in anterio-ventral development, including heart formation and positioning of the yolk sac (Thomas and Beddington, 1996; Bally-Cuif and Boncinelli, 1997; Tam and Behringer, 1997; Beddington and Robertson, 1998; Ruiz i Altaba, 1998). While in amphibian embryos the head and trunk organizer are spatially close, in the mouse they are clearly separated (Fig. 8).

An early indication of specialized patterning within the AVE came from the discovery of VE-1 (visceral endoderm-1), a serendipitously discovered antigen. At 5.0 days p.c., VE-1 is expressed in AVE overlying both embryonic and extraembryonic regions, while early in gastrulation this antigen is evident in AVE distal to the junction with the extraembryonic region (Rosenquist and Martin, 1995). The discovery of this AVE marker was followed by a series of *in situ* hybridization studies demonstrating that certain gene transcripts localize to AVE before and during early gastrulation. As discussed in detail below, many of these transcripts show dynamic patterns of expression, with expression in visceral endoderm heralding their subsequent expression in underlying ectodermal or mesodermal derivatives. Disrupting the expression of these genes through targeted mutagenesis impairs the development of the anterior central nervous system (CNS) or anterior mesoderm (Table I). These findings underscore the role of AVE as an early organizer specifying anterior identity prior to and early in gastrulation. Subsequently, anterior character is reinforced and maintained by expression of genes in node derivatives.

Visceral endoderm factors implicated in embryonic development and anterior patterning

A. Transcription factors

1. Evx-1

The mouse homeodomain gene *evx1* was originally isolated on the basis of homology to the *Drosophila even-skipped* gene

(Bastian and Gruss, 1990; Dush and Martin, 1992). Murine *evx1* is expressed in the visceral endoderm after implantation and prior to gastrulation (Spyropoulos and Capecchi, 1994), and later in cells of the embryonic ectoderm that give rise to the primitive streak. Subsequently, *evx1* mRNA is expressed in the posterior neuroectoderm and in the limb buds. Gene targeting studies have shown that Evx1 deficient mouse embryos implant in the uterine epithelium but fail to differentiate extraembryonic tissues or form egg cylinders (Spyropoulos and Capecchi, 1994). Embryonic death occurs shortly after implantation, and resorption of embryos is evident prior to the normal time of gastrulation. When cultured *in vitro*, trophoblast and epiblast tissues from mutant embryos grow briefly, but then degenerate. On the basis of these studies it has been suggested that *evx1* expression within visceral endoderm is critical for differentiation of the embryo. Gene targets for Evx-1 within visceral endoderm have not been established.

2. HNF-4

Another transcription factor expressed in visceral endoderm which has been implicated in the regulation of gastrulation is HNF-4 (also termed HNF-4α), a member of the steroid hormone superfamily of transcription factors (Duncan *et al.*, 1994). Recent evidence suggests that fatty acyl-CoA thioesters are ligands for this factor (Hertz *et al.*, 1998). HNF-4 has been conserved through evolution, with orthologs found in *Drosophila*, *Xenopus*, mouse, and human. Mammalian HNF-4 binds to the promoters of a variety of genes expressed in both visceral endoderm and liver, including *transthyretin* and *ApoCIII*. In the developing mouse embryo, expression of this transcription factor is first evident in visceral endoderm cells at 5.5 days p.c. (Duncan *et al.*, 1994). Later in gestation, HNF-4 mRNA is seen in the liver diverticulum and the gut endoderm (Duncan *et al.*, 1994). *Hnf4-/-* embryos initiate gastrulation, as evidenced by expression of the early mesodermal marker *Brachyury*. However, these homozygous deficient embryos fail to express later mesodermal differentiation markers or progress through gastrulation. As a result, the development of embryonic structures is severely impaired (Chen *et al.*, 1994). Excessive cell death is seen in the embryonic ectoderm as early as 6.5 days p.c.. Visceral endoderm forms in both *Hnf4-/-* embryos and *Hnf4-/-* ES cells differentiated *in vitro* into embryoid bodies. Furthermore, functional studies have shown that HNF-4 deficient endoderm is able to endocytose exogenously added horseradish peroxidase (Chen *et al.*, 1994). Despite the presence of a morphologically normal endodermal cell layer, aberrations in gene expression are seen in the visceral endoderm of HNF-4 deficient animals, including absent or decreased expression of *AFP*, *transferrin*, *apoAI*, *apoAIV*, *apoB*, and *transthyretin* (Duncan *et al.*, 1997).

Tetraploid complementation provides a useful tool to show that functional defects in visceral endoderm contribute to the defects in development of the embryo proper. When tetraploid cells, derived from electrofusion of 2-cell stage embryos, are aggregated with diploid *(2n)* ES cells or embryos, the tetraploid cells contribute efficiently to visceral endoderm and trophectoderm in the resultant chimeras, whereas the embryo proper is derived exclusively from diploid cells (Figs. 5,6) (Snow, 1975; Tarkowski *et al.,* 1977; Nagy *et al.*, 1993; James *et al.,* 1995).

Tetraploid complementation has been used to rescue the postgastrulation development of HNF-4 deficient embryos (Duncan

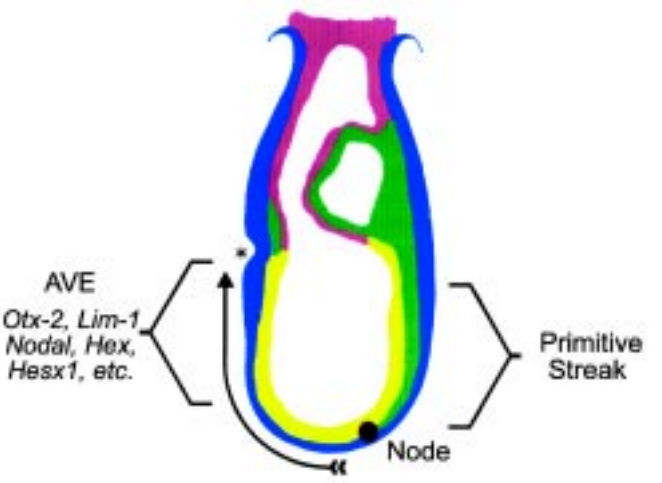

Fig. 3. Day 7.5 mouse embryo. *The parietal endoderm is not shown. The junction between extraembryonic and embryonic tissue is indicated by the asterisk. The left bracket highlights the AVE and some of the genes expressed in this region. The route of migration of visceral endoderm cells is indicated by the arrow. The color scheme is the same as that shown in Figure 1. Adapted from Kaufman, 1992; Thomas et al., 1998.*

et al., 1997). Unlike their non-chimeric counterparts, *4n Hnf4+/+ ÷ 2n Hnf4-/-* chimeric embryos undergo gastrulation, indicating that *Hnf4-/-* embryos are capable of completing gastrulation in the presence of wild type visceral endoderm. These genetic studies underscore the notion that functional visceral endoderm is required for normal gastrulation. Furthermore, these experiments suggest that HNF-4 controls the expression of certain genes in visceral endoderm which are critical for directing gastrulation in the adjoining germ layers.

Some potential target genes for HNF-4 in endoderm have emerged through studies of a human disease, known as maturity-onset diabetes of the young type 1 (MODY1), in which a truncated, loss-of-function mutant of human HNF-4 is expressed (Yamagata *et al.*, 1996; Stoffel and Duncan, 1997). This variant HNF-4 molecule does not bind DNA, dimerize, or *trans*-activate promoters (Yamagata *et al.*, 1996; Stoffel and Duncan, 1997). To identify the genes potentially affected by the human mutation, a panel of genes associated with insulin action has been examined in embryoid bodies derived from mouse *Hnf4* null embryonic stem cells. Among the genes down-regulated in mutant embryoid bodies are *glucose transporter 2*, *aldolase B*, *glyceraldehyde-3-phosphate dehydrogenase*, *intestinal specific fatty acid binding protein* and *CRABPs* (Stoffel and Duncan, 1997). These reductions are even more pronounced in HNF-4 deficient mouse embryos. Thus, the role of HNF-4 in the yolk sac endoderm may be to support the expression of genes which are subsequently expressed in analogous embryonic and adult organs as intestine, liver, and pancreas. The collapse of gastrulation in HNF-4 deficient mouse embryos may reflect "death from starvation" (Chen *et al.*, 1994; Copp, 1995; Duncan *et al.,* 1997)

In addition to the regulation of genes important for nutrient uptake and transport, studies in *Xenopus* suggest that HNF-4 may play a morphogenetic role in establishing mesodermal and endodermal lineages (Holewa *et al.*, 1996; Weber *et al.,* 1996). In early *Xenopus* development, a gradient of maternal HNF-4 is distributed from the animal to vegetal pole, allowing HNF-4 to

interact with different targets. One of the putative target genes in *Xenopus* is *HNF-1α*, which requires HNF-4 binding for Activin-A stimulated expression (Weber *et al.*, 1996).

3. Hex/Prh

Hex is a homeobox gene implicated in anterior patterning (Bedford *et al.*, 1993), also known as *Prh* (Compton *et al.*, 1992; Hromas *et al.*, 1993). This gene exhibits dynamic expression during early mouse development. *Hex* is first expressed in a small patch of visceral endoderm at the distal tip of the egg cylinder, one day before primitive streak formation (Bedford *et al.*, 1993; Thomas *et al.*, 1998). Lineage tracing studies indicate that these cells, while continuing to express *Hex*, move to assume an anterior position (Thomas *et al.*, 1998). Hence, Hex is an early marker of A-P asymmetry in the mouse embryo. Later in development, *Hex* is expressed in definitive endoderm and in the thyroid primordium. In addition, this gene is transiently expressed in endothelial and hematopoietic precursors in the blood islands of the yolk sac and in the endocardium. A phenotype for Hex deficient mice has not been reported yet.

4. Hesx1/Rpx

The homeobox gene *Hesx1* (Thomas and Beddington, 1996), also known as *Rpx* (Hermesz *et al.*, 1996), is first expressed at the start of gastrulation and is confined to a small domain of anterior visceral endoderm, distal to the embryonic/extraembryonic junc-

TABLE 1

MUTANT MOUSE PHENOTYPES THAT IMPLICATE VISCERAL ENDODERM IN DEVELOPMENT OF THE EMBRYO PROPER

Factor	Description	Consequences of gene disruption in the mouse
Evx1	Homeodomain transcription factor	Embryos implant but fail to differentiate extraembryonic tissues or form egg cylinders
HNF-4	Steroid hormone receptor superfamily member	Failure of gastrulation, decreased expression of visceral endoderm markers such as AFP, complemented in tetraploid chimeras
Otx-2	Homeodomain transcription factor	Abnormalities in anterior development, complemented in diploid ES cell chimeras
Lim-1	Lim-homeodomain transcription factor	Abnormalities in anterior development (headless embryos)
Goosecoid	Homeodomain transcription factor	Abnormalities in presphenoidal cranial bone and ribs
HNF3β	Winged helix transcription factor	Defects in dorsal-ventral patterning, ectopic yolk sac
Goosecoid-/-:: HNF3β+/-	Compound deficiency of the above two factors	Severe growth defects, absence of optic vesicles, abnormalites in A-P and D-V patterning, decreased expression of Shh, Hnf3β, and Fgf8
Hesx1	Homeodomain transcription factor	Anterior CNS defects and pituitary dysplasia
GATA-4	Zn finger transcription factor	Defects in ventral morphogenesis, abnormal foregut formation, failure of fusion of the bilateral myocardial primordia, ectopic yolk sac, complemented in diploid ES cell chimeras
GATA-6	Zn finger transcription factor	Failure of gastrulation, decreased expression of visceral endoderm markers such as HNF-4, AFP, and GATA-4, complemented in diploid ES cell chimeras
Smad4/DPC4	Tumor suppressor gene product, associates cytoplasmic tails of TGFβ receptor superfamily members	Gastrulation failure, abnormal visceral endoderm morphology, with anterior truncations, complemented in tetraploid chimeras
Smad2	Phosphoprotein, heterodimerizes with Smad4	Gastrulation failure, lack of proper columnar/squamous differentiation in visceral endoderm, complemented in diploid ES cell chimeras
Nodal	TGFβ growth factor superfamily member	Gastrulation defects, abnormalities in anterior neuroectoderm development, complemented in diploid ES cell chimeras
ActRIB	Type I Activin receptor	Gastrulation defects, lack of proper columnar/squamous differentiation in visceral endoderm, partially complemented in diploid ES cell chimeras
Furin	Protease which processes precursors of TGFβ family members	Defects in ventral morphogenesis, failure of fusion of the bilateral myocardial primordia, disorganized yolk sac vessels
Cripto	Member of the EGF-CGC gene family	No formation of primitive streak or embryonic mesoderm, thickened distal visceral endoderm, absence of posterior neuroectoderm (head without a trunk)
Hβ58	Protein whose yeast ortholog is involved in the secretory pathway	Embryos arrest at 8.5 days p.c., all three germ layers are present
Huntingtin	Huntington's disease gene	Die shortly after gastrulation, disorganized embryo proper, abnormal visceral endoderm morphology, complemented in diploid ES cell chimeras

tion. Approximately one day after *Hesx1* transcripts are detected in endoderm, expression is evident in the adjacent anterior ectoderm fated to become prosencephalon. Later, expression becomes restricted to Rathke's pouch, the primordium of the anterior pituitary gland. This portion of the neuroectoderm continues to express *Hesx1* over the ensuing days of development. Physical removal of endoderm cells expressing *Hesx1* from cultured mouse embryos during gastrulation either prevents or severely diminishes the later expression of *Hesx1* in anterior neuroectoderm, but does not affect gene expression in the midbrain or more caudal regions of the developing central nervous system (Thomas and Beddington, 1996). Thus, an interaction between anterior primitive endoderm and the adjacent ectoderm appears to influence anterior neural plate identity. These results led investigators to propose that the visceral endoderm is responsible for the initial induction of rostral identity in adjacent ectoderm and that subsequently this identity is reinforced by axial mesoderm or endoderm, when visceral endoderm is displaced from the embryo proper (Thomas and Beddington, 1996). Mice lacking Hesx1 exhibit anterior CNS defects and pituitary dysplasia (Dattani *et al.*, 1998). Specifically, the mutant embryos have a reduced prosencephalon, anopthalmia or micropthalmia, defective olfactory development, and bifurcations in Rathke's pouch. Animals surviving to the neonatal period exhibit abnormalities in the corpus callosum, the anterior and hippocampal commissures, and the septum pellucidum. A comparable phenotype is seen in humans with septo-optic dysplasia (SOD), and individuals with SOD have been shown to have missense mutations within the human HESX1 homeodomain which abrogate DNA-binding.

5. Otx-2

Mouse *Otx2* is a *bicoid*-class homeobox gene that is related to the *Drosophila orthodenticle* gene (Royet and Finkelstein, 1996). At 5.5 days p.c. *Otx2* mRNA is expressed diffusely in both the epiblast and the visceral endoderm. From the early primitive streak to headfold stages, the expression of *Otx2* becomes restricted to the anterior end of the embryo proper and the underlying endoderm. Tissue recombination experiments indicate that restriction of *Otx2* expression to the anterior region depends on the negative signals from posterior mesendoderm as well as positive signals from anterior mesendoderm (Ang *et al.*, 1994). *Otx2-/-* embryos exhibit severe defects in forebrain and midbrain development (Acampora *et al.*, 1995; Matsuo *et al.*, 1995; Ang H.L. *et al.,* 1996). Interestingly, *Otx2* heterozygotes also display an increased incidence of craniofacial defects (Matsuo *et al.*, 1995).

Analysis of chimeric embryos derived from injection of *Otx2-/-* ES cells into blastocysts has been used to demonstrate an essential function for *Otx2* in the visceral endoderm (Matsuo *et al.*, 1995; Rhinn *et al.*, 1998). Since ES cells injected into blastocysts contribute poorly to visceral endoderm (Beddington and Robertson, 1989), the visceral endoderm component of these chimeric embryos is almost exclusively wild type, while the embryo proper contains *Otx2-/-* cells. In these chimeras the anterior neural plate forms without expressing *Otx2*, but there is decreased expression of critical regulatory genes, including *Hesx1*, *Wnt1*, *En*, and *Pax2,* in the neural plate. These studies indicate that expression of *Otx2* in visceral endoderm influences gene expression in adjoining ectoderm.

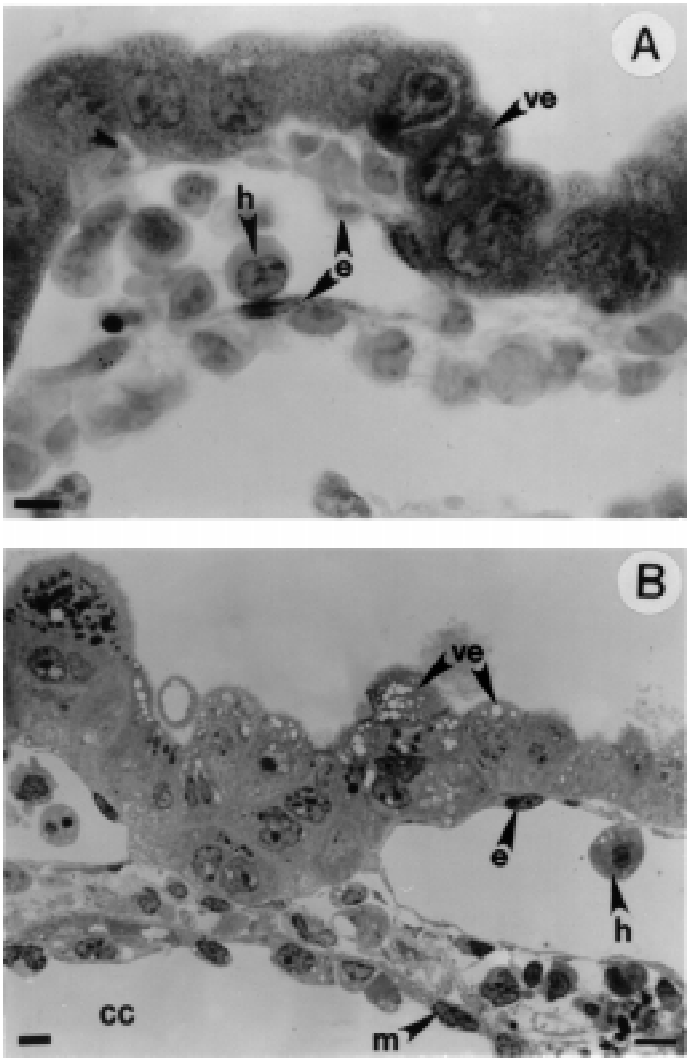

Fig. 4. Blood island formation *in vivo* and *in vitro*. *Sections through* **(A)** *the visceral yolk sac of a 9 day p.c. embryo, and* **(B)** *the surface of a day 10 cystic embryoid body. Note the intimate association between visceral endoderm and blood islands. Abbreviations: cc, cystic cavity of the embryoid body; e, endothelial cell; h, hematopoietic cell; m, mesothelial cell; ve, visceral endoderm. Bars, 10 μm.*

Experiments in which the *lacZ* gene was inserted into the *Otx2* locus (Acampora *et al.*, 1995) suggest that Oxt2 expression in visceral endoderm affects expression of Otx2 itself in underlying anterior ectoderm. In wild type 6.5 day p.c. embryos the amount of *Otx2* mRNA expression in the epiblast is similar to that observed in the endoderm layer, but in *Otx+/-LacZ* embryos the amount of LacZ expression in the epiblast is considerably lower than in the visceral endoderm. This discrepancy between visceral endoderm and ectoderm expression is even more pronounced in homozygous embryos. Taken together, these experiments suggest that there is a requirement for normal expression of *Otx2* in the anterior visceral endoderm for the embryo proper to express *Otx2* appropriately in the anterior neuroectoderm.

Recent studies with homozygous deficient embryos in which the *Otx2* coding sequence was replaced with sequence encoding Otx1, a factor normally expressed in neural tissue but not AVE, indicate that Otx-1 can substitute for Otx-2 in specification and

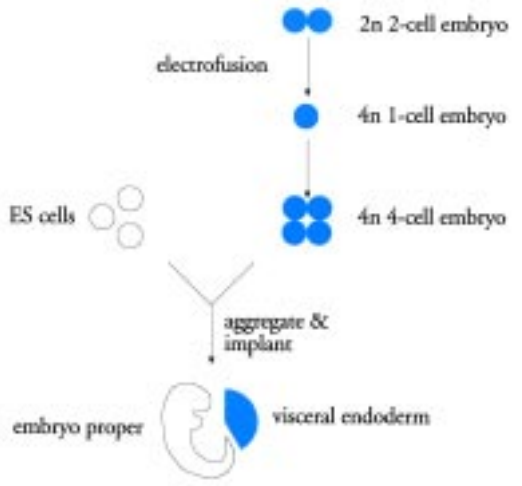

Fig. 5. **Generation of tetraploid aggregation chimeras.** *Tetraploid (4n) embryos, produced by electrofusion of diploid (2n) 2-cell stage embryos, are aggregated with ES cells. These aggregates are then implanted in pseudopregnant females. In the resultant embryos, tetraploid cells contribute to visceral endoderm (and trophectoderm derivatives), but not to the embryos proper. To facilitate lineage tracing, embryos bearing a lacZ transgene (blue) can be used to generate the tetraploid cells.*

initial patterning of the neural plate and organization of the primitive streak (Acampora *et al.*, 1998; Suda *et al.*, 1999). However, the rostral brain fails to develop in these embryos. While Otx1 mRNA is detected in both visceral endoderm and the epiblast of these "knock-in" embryos, protein is evident only in the visceral endoderm due to differences in translation in embryonic and extraembryonic tissues (Acampora *et al.,* 1998). On the basis of these findings it has been suggested that early induction of anterior neural patterning is regulated by Otx-2 in visceral endoderm, while subsequent maintenance of forebrain-midbrain identity is mediated by epiblast-derived cells.

6. Lim-1

The LIM-homeobox gene *Lim1* is expressed in the anterior visceral endoderm during early gastrulation (Belo *et al.*, 1997; Tam and Behringer, 1997). Subsequently, expression is seen in the primitive streak and its derivatives as well as in the mesodermal wings and prechordal plate (Shawlot and Behringer, 1995). Lim-1 deficiency results in embryonal death with a morphological phenotype strikingly similar to that of *Otx-2* mutants. During early gastrulation, mutant embryos do not form a recognizable node, head process, or prechordal mesoderm. At later stages, the embryos are characterized by a complete lack of anterior head structures. The extraembryonic and embryonic regions were separated by a constriction, which disrupts the normal positioning of the embryo relative to the yolk sac (Shawlot and Behringer, 1995). It has been speculated that the truncated head structures observed in the *Lim1-/-* embryos, like *Otx2-/-* mutants, result from the alteration of gene expression in the anterior visceral endoderm (Belo *et al.*, 1997).

7. HNF-3β

This transcription factor, a member of the winged-helix family, is abundantly expressed in the visceral endoderm of the 6 day p.c. embryo; later this message is expressed in the node, notochord, floor plate, prechordal mesoderm, and the definitive endoderm of the gut (Ang and Rossant, 1994; Copp, 1995; Farrington *et al.*, 1997). *HNF3β-/-* mouse embryos die between 6.5 and 9 days p.c. and display growth failure and a spectrum of developmental defects (Ang and Rossant, 1994; Weinstein *et al.,* 1994; Farrington *et al.*, 1997; Manova *et al.*, 1998). There is increased apoptotic cell death in the distal embryonic ectoderm, although this is not as pronounced as that seen in *Hnf4-/-* embryos (Manova *et al.*, 1998). Additionally, *HNF3β-/-* embryos exhibit an absence of the node and notochord, which contributes to secondary defects in dorso-ventral (D-V) patterning of the neural tube. Definitive endoderm cells form, but foregut morphogenesis is abnormal and the endoderm cells remain on the external surface of the embryo. Although *HNF3β-/-* embryos show severe defects in D-V patterning due to the loss of an organized node and axial mesoderm cells, patterning of the neural tube along the A-P axis occurs in mutant embryos, from the midbrain to the posterior end of the spinal cord (Ang and Rossant, 1994; Weinstein *et al.*, 1994). In a minority of embryos, A-P patterning is disrupted, manifested as underdevelopment of the rostral head structures.

In addition to these other morphologic abnormalities, *HNF3β-/-* embryos often display aberrant connections between the yolk sac

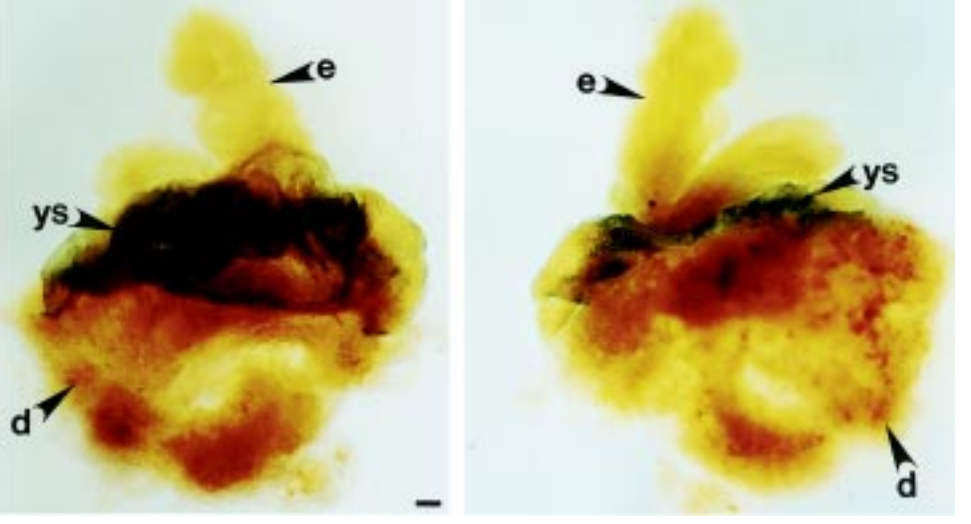

Fig. 6. Whole-mount X-gal staining of an 8 day p.c. chimeric mouse embryo produced by aggregation of ES cells with tetraploid cells derived from a *Rosa26* transgenic mouse. *Ventral* **(left)** *and dorsal* **(right)** *views of the same embryo are shown. The Rosa26 line bears a lacZ transgene that is ubiquitously expressed (Friedrich and Soriano, 1992). ES cell and tetraploid derivatives can be discriminated on the basis of X-gal staining. Note that tetraploid cells (blue) contribute to visceral yolk sac endoderm but not to the embryo proper. Abbreviations: d, decidua; e, embryo proper; ys, yolk sac. Bar, 100 μm.*

and embryo (Farrington *et al.*, 1997; Manova *et al.*, 1998), similar to those seen in Otx2-/- and Lim1-/- embryos. The site of attachment is variable, with some yolk sacs connected along the presumptive ventral surface and others attached more posteriorly such that the anterior end of the embryo protrudes out of the yolk sac and away from the ectoplacental cone. The visceral endoderm layer appears histologically normal, although expression of AFP and transthyretin are decreased in *HNF3β-/-* yolk sacs (Farrington *et al.*, 1997). These findings indicate that the movements responsible for repositioning the embryo relative to the yolk sac do not occur properly in *HNF3β* mutants, possibly due to defects intrinsic to visceral endoderm cells, such as abnormalities in cell adhesion and/or cell migration. In chimeras generated by aggregating wild type tetraploid embryos with *HNF3β-/-* ES cells the constriction phenotype is rescued, and there is proper elongation of the primitive streak (Dufort *et al.*, 1998). However, this restoration of streak elongation is not sufficient to allow formation of the node or notochord in the *HNF3β* mutants. The chimeras lack foregut and midgut endoderm, and exhibit abnormalities in left-right asymmetry. Therefore, HNF-3β, like Otx2, appears to have distinct functions in visceral endoderm and epiblast. In visceral endoderm HNF-3β promotes streak morphogenesis, while in epiblast it helps to specify the node and notochord.

8. Goosecoid

The distribution and function of HNF-3β are intertwined with a homeobox gene product, Goosecoid. The *Goosecoid (gsc)* and *HNF3β* genes are co-expressed in several regions, including the visceral endoderm, all three germ layers of the anterior primitive streak, and the rostral end of the gastrulating mouse embryos (Belo *et al.*, 1997; Filosa *et al.*, 1997). Gsc-deficiency in mice does not affect A-P patterning of the neural tube, but results in craniofacial (presphenoidal cranial bone) and rib abnormalities, consistent with the later domains of expression of this gene (Rivera-Perez *et al.*, 1995). Functional redundancy between Goosecoid and HNF-3β has been evoked to account for the lack of phenotype affecting the neural tube in *gsc-/-* embryos and tested by generating double-mutant mice. Embryos of genotype *gsc-/-*::*HNF3β+/-* show a distinctive phenotype, which is evident as early as 8.75 days p.c. (Filosa *et al.*, 1997). These double-mutant embryos exhibit defects in anterior neuroectoderm development (i.e., decreased forebrain size), as well as abnormalities in the branchial arches, heart looping, and the gut endoderm. Loss of expression of both *HNF3β* and *Shh* is observed in the notochord and ventral neural tube of these embryos. These results indicate that Goosecoid and HNF-3β interact to regulate *Shh* expression and thereby influence D-V patterning in the neural tube, and that interaction between Goosecoid and HNF-*3β* regulates other signaling molecules required for proper development of the foregut, branchial arches, and heart.

9. GATA-4

The zinc finger transcription factor GATA-4 belongs to a family of structurally related proteins that recognize the consensus DNA sequence (A/T)GATA(A/G), present in the promoters or enhancers of a variety of genes, including genes expressed selectively in cardiomyocytes and endoderm derivatives (Arceci *et al.,* 1993; Grépin *et al.,* 1994, 1995, 1997; Bielinska and Wilson, 1995; Durocher *et al.*, 1997; Evans, 1997; Hasegawa *et al.,* 1997; Herzig *et al.,* 1997; Kuo *et al.,* 1997; Molkentin *et al.,* 1997; Narita

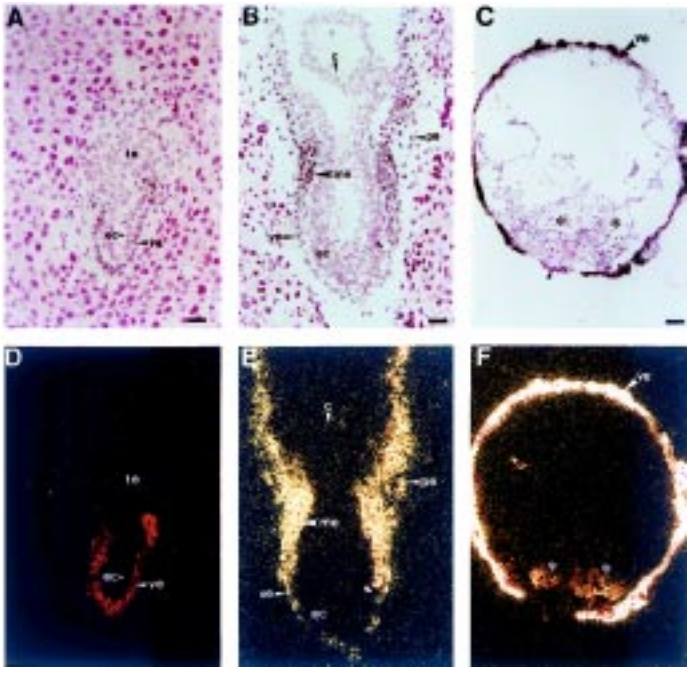

Fig. 7. *In situ* hybridization of GATA-4 mRNA in mouse embryos and ES cell-derived embryoid bodies (C,F). *Corresponding bright field* **(A-C)** *and dark field* **(D-F)** *views of sections through a 6 day p.c. embryo (A,D), 7 day p.c. embryo (B,E), and 10 day cystic embryoid body (C,F) are shown. GATA-4 mRNA is abundantly expressed in the visceral endoderm and nascent mesoderm of the embryos. This pattern of expression is recapitulated in the embryoid body. Abbreviations: c, chorion; ec, ectoderm; me, mesoderm; te, trophectoderm; pe; parietal endoderm; ve, visceral endoderm. Bar, 30 μm.*

et al., 1997a; Bossard and Zaret, 1998). GATA-4, which has been implicated in the hypertrophic response of cardiomyocytes to stress (Durocher *et al.*, 1997; Hasegawa e*t al.*, 1997; Herzig *et al.*, 1997; Molkentin *et al.*, 1998), interacts with other factors that modulate transcriptional activity, including CREB-binding protein (Blobel *et al.*, 1998), Nkx-2.5 (Durocher *et al.*, 1997), and NF-AT3 (Molkentin *et al.*, 1998). During embryonic development GATA-4 is expressed in precardiac splanchnic mesoderm and adjacent endoderm during the active processes of foregut invagination and heart tube formation (Fig. 7) (Heikinheimo *et al.*, 1994; Kuo *et al.*, 1997; Molkentin *et al.*, 1997). This pattern of expression in visceral endoderm and select epiblast derivatives is recapitulated in embryoid bodies (Fig. 7).

Gata4-/- ES cells exhibit a defect in visceral endoderm formation during *in vitro* differentiation into embryoid bodies (Soudais *et al.*, 1995). Consequently, expression of many visceral endoderm markers is reduced in these embryoid bodies. This *in vitro* defect in visceral endoderm differentiation can be overcome by the addition of RA to the culture media (Bielinska and Wilson, 1997). The reappearance of visceral endoderm on the surface of RA-treated GATA-4 deficient embryoid bodies is accompanied by elevated expression of GATA-6 (Bielinska and Wilson, 1997), suggesting the possibility of interplay/redundancy among these GATA-binding proteins.

Gata4-/- mice die before 9.5 days p.c. (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). Some of these mutant embryos do not survive the early postimplantation period, possibly secondary to

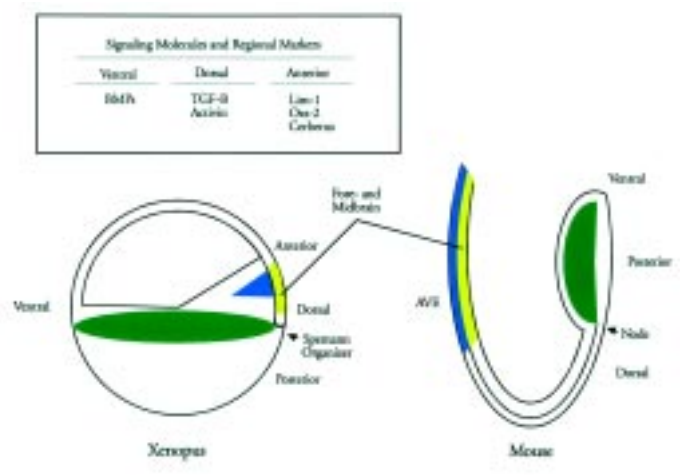

Fig. 8. **Anterior organizer regions in *Xenopus* and mouse embryos.** *Different colors designate functionally analogous cell layers. Blue, deep mesendodermal cells in the frog and AVE in the mouse; yellow, anterior neuroectoderm; green, nonaxial mesoderm. Adapted from Bouwmeester and Leyns (1997).*

defects in primitive endoderm function (Molkentin *et al.*, 1997). In the embryos progressing through gastrulation visceral endoderm cells are present, and express elevated levels of GATA-6 mRNA (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). *Gata4-/-* embryos display profound defects in ventral morphogenesis: cardiomyocytes are present but bilateral myocardial primordia fail to migrate and fuse ventrally, resulting in formation of two dorsolateral heart tubes (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). In addition, the foregut is disorganized in appearance, the pericardial cavity is absent, and the yolk sac and amnion are displaced dorsally. Reminiscent of *HNF3β-/-* and *Lim1-/-* embryos (Shawlot and Behringer, 1995; Farrington et al., 1997), GATA-4 deficient embryos lie outside the yolk sac, reflecting aberrant connection between the yolk sac and embryo. Differentiation markers for cardiomyocytes (e.g., cardiac α-myosin heavy chain, atrial natriuretic factor), visceral endoderm (e.g., AFP, HNF-4), and anterior neuroectoderm (e.g., Otx-2) are normally expressed in *Gata4-/-* embryos (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). These findings suggest that expression of GATA-4 in cardiogenic splanchnic mesoderm, associated endoderm, or both of these tissues is essential for ventral patterning.

A chimeric embryo approach has been used to demonstrate that GATA-4 expression in the visceral endoderm is required for ventral morphogenesis (Narita *et al.*, 1997a,b). Introduction of *Gata4-/-* ES cells into wild type 8-cell stage embryos produces chimeric embryos in which the visceral endoderm is composed of wild type cells. High percentage null chimeras (8-10 days p.c.) in which *Gata4+/+* cells are restricted to visceral yolk sac endoderm and small portions of the foregut/hindgut endoderm exhibit normal development of the heart, foregut, and surrounding tissues, suggesting that expression of GATA-4 in endoderm is essential for ventral morphogenesis.

Studies on other mammals suggest that the presence of dorsolateral, well-differentiated heart tubes in *Gata4-/-* mouse embryos may reflect delayed foregut closure and an abnormally shaped yolk sac rather than a defect in the promyocardium per se. Bilateral endocardial tubes and bilateral myoepicardial differentiation of the splanchnopleura are seen in species with a large yolk sac, relatively late closure of the foregut, and a broad anterior intestinal portal (AIP) (e.g., *Tupaia belangeri*, ferret, cat, rabbit) (Kuhn and Liebherr, 1987). In species with a small yolk sac and/or reversal of germ layers (e.g., mouse, rat, human), the foregut and AIP are formed earlier, and the heart primordium reaches its median position ventral to the foregut before significant myocardial differentiation has occurred (DeRuiter *et al.*, 1992).

10. GATA-6

Another member of the GATA-binding family of transcription factors, GATA-6, has also been implicated in visceral endoderm function. The tissue distribution of GATA-6 partially overlaps that of GATA-4, with expression of GATA-6 evident in visceral endoderm, parietal endoderm, cardiomyocytes, gut epithelium, bronchial epithelium, and selected other cell types (Laverriere *et al.*, 1994; Morrisey *et al.,* 1996; Narita *et al.,* 1996; Evans, 1997; Heikinheimo *et al.*, 1997;). Targeted disruption of the *Gata6* gene in mice results in early embryonic death between 5.5 and 7.5 days p.c. (Morrisey *et al.*, 1998; Koutsourakis *et al.*, 1999). GATA-6 deficient embryos exhibit growth retardation and excessive apoptosis in epiblast tissue. The mutant embryos exhibit patches of morphologically recognizable but functionally defective visceral endoderm, with reduced expression of GATA-4 and HNF-4 (Morrisey *et al.*, 1998). Regions lacking a well developed visceral endoderm layer also show defects in underlying ectoderm (Koutsourakis *et al.*, 1999). *Gata6-/-* blastocysts cultured *in vitro* exhibit normal trophoblast outgrowth but impaired growth of the inner cell mass (Koutsourakis *et al.*, 1999). Like GATA-4 deficient embryoid bodies (Soudais *et al.*, 1995), GATA-6 deficient embryoid bodies lack a surface layer of visceral endoderm (Morrisey *et al.*, 1998). Moreover, *Gata6-/-* embryoid bodies are deficient in expression of GATA-4, AFP, and HNF-4. Injection of *Gata6-/-* ES cells into wild type blastocysts produces high percentage GATA-6 deficient chimeras in which the visceral endoderm is composed exclusively of wild type cells. These embryos undergo normal gastrulation. In contrast, injection of wild type ES cells into *Gata6-/-* blastocysts gives rise to chimeric embryos in which the visceral endoderm is composed exclusively of GATA-6 deficient cells, and these embryos die shortly after implantation (Koutsourakis *et al.*, 1999). These results suggest a defect in primitive endoderm and/or visceral endoderm function, leading to subsequent starvation of or inadequate trophic support for underlying epiblast. GATA-6 may be an early factor required for proper differentiation of visceral endoderm.

The involvement of GATA-binding transcription factors in ventral development has been conserved through evolution. Injection of *Xenopus* embryos with an RNA encoding a dominant-interfering GATA factor, consisting of the zinc finger domain of GATA-2 fused to the repressor domain of *Drosophila* engrailed protein, alters ventral development (Sykes *et al.*, 1998). Injection of this RNA ventrally induces formation of incomplete secondary axes, lacking head and notochord, but containing a neural tube, ectopic muscle, and an ectopic gut lumen. However, inhibiting GATA activity in the ectoderm does not lead to neuralization. On the molecular level, this dominant inhibitor suppresses expression of *Wnt-8* and the homeobox gene *Vent-1* but not *Vent-2* or *BMP-4*. Chordin, a BMP-antagonist secreted by the organizer (Sasai *et*

al., 1995; Piccolo *et al.,* 1996), is ectopically expressed. Coinjection of GATA-1, GATA-2, or GATA-6 mRNA can rescue the phenotype. These findings suggest that the dominant-interfering factor disrupts the function of one or more GATA-binding proteins essential for ventral cell fate. Whether GATA-4, GATA-6, or another member of this family is critical for ventral fate in *Xenopus* is still unknown.

11. Msg1 and Mrg1

The *melanocyte specific*-1 *(Msg1)* and *Msg1-related-1 (Mrg1)* genes are members of a newly recognized gene family (Shioda *et al*., 1996; Dunwoodie *et al*., 1998). Features of the proteins encoded by these genes suggest that they function as transcription factors. Msg1 antigen is found in the nucleus (Shioda *et al*., 1996) and both Msg1 and Mrg1 contain amino acid sequences which, when fused to the *GAL4* DNA-binding domain, activate transcription in mammalian cells (Shioda *et al*., 1997).

Msg1 and *Mrg1* exhibit distinct but partially overlapping expression patterns, not restricted to melanocytes (Dunwoodie *et al*., 1998). Prior to gastrulation, *Msg1* is expressed in the distal and proximal visceral endoderm. By the primitive streak stage, expression in the embryonic visceral endoderm is asymmetrical, extending more toward the posterior side of the egg cylinder. As development proceeds, *Msg1* expression is seen in the horseshoe-shaped cardiogenic plate. During fusion of the primordial heart tubes, *Msg1* expression becomes restricted to the posterioventral aspect of the linear heart tube, which gives rise to the left ventricle. Later in embryogenesis, some expression of *Msg1* can be detected in the right ventricle and atria as well as the posteriodorsal limb mesoderm and sclerotome. In the adult, *Msg1* is expressed only in heart and testes. Before the onset of gastrulation, *Mrg1* is expressed in a domain of the AVE, abutting the extraembryonic junction. Shortly thereafter, *Mrg1* expression is evident in the underlying rostral mesoderm of the embryo, which will go on to form the heart and septum transversum. This coincidental expression in visceral endoderm and rostral mesoderm resembles the spatiotemporal expression of GATA-4, GATA-6, and furin (see below), supporting the notion that aspects of mesodermal identity originate in the visceral endoderm.

B. Growth factors and signaling molecules

1. Nodal

Mouse Nodal is a member of the TGF-β superfamily of growth and differentiation factors. Nodal is a secreted protein produced by both endodermal and ectodermal derivatives (Zhou *et al*., 1993). In the pregastrulation embryo abundant *nodal* expression is seen in the posterior region of the epiblast, where the primitive streak forms (Varlet *et al*., 1997). Posterior expression persists during the initial stages of streak formation and is then down-regulated as the streak elongates. Thereafter, *nodal* expression is seen in a subset of node progenitors and then in the edges of the notochordal plate. In addition to this ectodermal expression pattern, *nodal* is transiently expressed throughout the visceral endoderm prior to and during primitive streak formation (Varlet *et al*., 1997). Mouse mutants deficient in *nodal* do not initiate primitive streak formation and die at the gastrulation stage (Conlon *et al*., 1994). These mutant embryos lack a morphologically distinct visceral yolk sac, allantois, amnion, or a definitive fetal portion.

A chimeric embryo approach has been used to demonstrate that Nodal expression in visceral endoderm is required for normal development of the anterior axis (Varlet *et al*., 1997). Injection of wild type ES cells into *nodal-/-* blastocysts gives rise to chimeric embryos in which the visceral endoderm is composed exclusively of Nodal deficient cells, whereas introduction of *nodal-/-* ES cells into wild type blastocysts produces chimeric embryos in which the visceral endoderm is composed of wild type cells. Through analysis of these chimeras, investigators have concluded that Nodal signaling in the ectoderm is necessary for primitive streak formation, since the gastrulation defect in nodal-deficient embryos can be rescued by the inclusion of small numbers of wild type cells. Chimeric embryos composed of Nodal deficient visceral endoderm fail to develop rostral neural structures, including the forebrain and anterior midbrain, suggesting that *nodal* expression by visceral endoderm is critical for patterning the anterior aspects of the A-P axis, analogous to observations made on the transcription factors Otx-2 and Lim-1.

2. Activin receptor ActRIB

This molecule is a type I transmembrane serine/threonine kinase receptor that forms heteromeric complexes with type II activin receptors, thereby mediating signaling by activin and possibly other members of the TGF-β superfamily (Massagué, 1996; Massagué *et al*., 1997). Upon ligand binding the type II receptors phosphorylate type I receptors, such as ActRIB, which in turn phosphorylate downstream mediators such as Smad2 (see below). During mouse gastrulation *ActRIB* mRNA is expressed in epiblast, extraembryonic ectoderm, and, more weakly, in proximal visceral endoderm (Gu *et al*., 1998). Targeted disruption of the *ActRIB* gene results in pre-gastrulation lethality (Gu *et al*., 1998). The mutant embryos are disorganized, lack a primitive streak, and do not express mesodermal markers such as *Brachyury*. Columnar visceral endoderm cells, but not distal squamous endoderm cells, are seen in these embryos. Chimera analysis has been used to show that ActRIB expression in visceral endoderm is required for normal gastrulation (Gu *et al*., 1998). Injection of wild type ES cells into *ActRIB-/-* blastocysts results in embryos in which the visceral endoderm is composed exclusively of *ActRIB-/-* cells, while wild type cells contribute to the epiblast and its derivatives. In these chimeras some extraembryonic mesoderm is seen, but all other derivatives of the epiblast fail to form. This suggests that visceral endoderm expression of the ActRIB receptor is critical for interactions between this cell layer and the subjacent epiblast.

Intriguingly, mice lacking Activins A and B develop to term with no gross morphological defects (Matzuk *et al*., 1995a,b). This observation, coupled with the *ActRIB-/-* phenotype, suggests that ActRIB may recognize other ligands in the TGF-β superfamily, which alone or in combination with other factors are essential for gastrulation. Nodal is one of the potential ligands for this receptor (Gu *et al*., 1998).

3. Cerberus and cerberus-like

An extracellular signaling molecule involved in induction of the most anterior structures, termed Cerberus, has been characterized in *Xenopus.* This factor is expressed early during gastrulation in the yolky, endomesodermal cells in the deep layer of the Spemann organizer, a region which bears functional similarity to the anterior visceral endoderm of the mouse (Bouwmeester *et al*., 1996; Belo *et al.,* 1997). Fluorescent labeling shows that descendants of the cerberus-expressing region can be traced to the

endodermal cells of the foregut, the liver diverticulum, the anterior midgut, and the heart. When injected into specific regions of the blastomere, *Xcer* message causes duplication of the A-P axis, including an ectopic head, rotary cement glands, ectopic eyes, a secondary heart beating with its own pace, and a secondary liver (Bouwmeester *et al.*, 1996). However, Xcer cannot restore trunk-tail structures in the UV-treated embryos.

A related factor, termed Cerberus-like (Cer-l), mouse Cerberus-1 (mCer-1), or Cerberus-related (Cerr1) (Belo *et al.*, 1997; Biben *et al.,* 1998; Shawlot *et al.*, 1998), has been identified in the mouse. Like Xcer, mCer-1 contains a cysteine knot, characteristic of other signaling molecules including TGFβs and BMPs (Isaacs, 1995). With the exception of the cysteine-rich domain, the overall amino acid homology between *Xenopus* and murine Cerberus is relatively low, suggesting that these two factors may not be true orthologs. Expression of *mCer-l* is observed in the AVE before gastrulation, extending to the distal tip in early primitive streak stage. Between the midstreak and early headfold stages, expression is restricted to a small patch of endodermal cells. Later in development, *mCer-1* is expressed in endoderm overlying the neural plate proximal to the node, which includes anterior gut endoderm, and in mesoderm from prechordal plate and notochord, but not in the node or precardiac mesoderm. Finally, expression is evident in developing somites (Biben *et al.,* 1998). This pattern differs from the expression of *Xcer*, which is not found in mesoderm. Mouse explant experiments show that early presomitic mesoderm, but not later stages of mesoderm, can induce neural differentiation in anterior ectoderm (Shawlot *et al.,* 1998), providing circumstantial evidence that mCer-l can function as a neural signaling molecule. The pattern of expression of *mCer-l* in "headless" *Lim1-/-* and *Otx2-/-* embryos provides additional evidence that mCer-1 functions as an inducer of anterior neuroectoderm. In *Lim1-/-* embryos, expression of *mCer-l* in the AVE is markedly diminished (Shawlot *et al.*, 1998). In *Otx2-/-* embryos, which exhibit a constriction between embryonic/extraembryonic region, expression of *mCer-1* is confined to visceral endoderm at the distal tip (Biben *et al.*, 1998), while in the embryos whose visceral endoderm function has been partially restored by forced expression of Otx1, *mCer-1* is expressed normally (Acampora *et al.*, 1998). These findings suggest that AVE cells may be incorrectly specified in *Lim1-/-* and Otx2-/- embryos or that interactions between germ layers are required for the maintenance of *mCer-l* expression.

While homozygous null *mCer-1* mutants have not yet been characterized, the role of *mCer-1* can be inferred from experiments in which this transcript was ectopically expressed in *Xenopus* embryos. The neuralizing effect in embryos injected with *mCer-l* mRNA is less pronounced than in the case of *Xcer* mRNA; no cement gland or head-like structures are formed, and *mCer-l* does not block mesoderm formation (Belo *et al.*, 1997; Biben *et al.,* 1998). However, in animal cap assays both *Xcer* and *mCer-l* induce expression of the pan-neuronal marker N-CAM (Kintner and Melton, 1987) and the anterior/midbrain marker Otx2, but not hindbrain markers. The pan-endodermal marker, endodermin (Sasai *et al.*, 1996), is also induced. Based on studies in *Xenopus*, it has been proposed that the default fate of ectoderm is neural; unless antagonistic signals are received, the ectoderm will assume a dorsal, neural character (Hemmati-Brivanlou and Melton, 1997; Sasai and de Robertis, 1997). These antagonistic signals come from the ventrally expressed factors, such as BMPs and Wnts. The induction of a complete head-containing secondary axis depends on inhibition of both the BMP and Wnt signaling pathways, and *Xenopus* Cerberus inhibits both (Glinka *et al.,* 1997). In *Xenopus* animal cap assays, expression of BMP-4 induced mesodermal markers, such as globins, α-actin, and XeHand, is reduced by co-expression of Xcer or mCer-1 (Belo *et al.,* 1997; Biben *et al.*, 1998). The effect is mutually antagonistic, since the neural and endodermal markers induced by Xcer or mCer are overridden by BMP-4, especially in the case of mCer-1. Thus, while Xcer appears to be sufficient for head induction, mCer-1 appears to be a weaker inducer of head development in *Xenopus*.

5. Smads

Smads and their orthologs have been identified in a variety of organisms, including *Drosophila* (Raftery *et al.*, 1995; Sekelsky *et al.*, 1995), *C. elegans* (Savage *et al.*, 1996), *Xenopus*, mouse, and human, where these factors serve as intracellullar transducing molecules in TGF-β superfamily signaling pathways (Lagna *et al.*, 1996; Heldin *et al.,* 1997; Liu *et al.,* 1997; Massagué *et al.*, 1997; Nakao *et al.*, 1997). Human *SMAD4* is a tumor suppressor gene, also known as DPC4 (Deleted in Pancreatic Cancer) (Lemoine, 1997; Zhang *et al.*, 1997). Studies in *Xenopus* have shown that Smad4 forms a heterodimer with phosphorylated Smad1 in response to BMP signaling, and this complex participates in the induction of ventral mesoderm, while a Smad4-Smad2 complex is involved in dorsal signaling by TGF-β and Activin (Baker and Harland, 1996; Graff *et al.*, 1996; Abdollah *et al.,* 1997; Kretzschmar *et al.*, 1997; Massagué *et al.*, 1997; Souchelnytskyi *et al.,* 1997). The active complexes move to the nucleus, where they bind to specific genes (Chen *et al.*, 1997; Liu *et al.*, 1997; Weisberg *et al.*, 1998).

a) Smad4

In the mouse, *Smad4* mRNA is present in the early embryonic and extraembryonic region, including visceral endoderm. This mRNA is also detectable in cultured ES cells (Sirard *et al.*, 1998; Waldrip *et al.*, 1998). While heterozygous mutant embryos appear normal, homozygous *Smad4* null embryos die before 7.5 days p.c.. These embryos are small and show severe malformation of both the embryonic and extraembryonic regions (Sirard *et al.*, 1998; Yang *et al.*, 1998). In 6.5-7.0 day p.c. homozygotes, there is a block in gastrulation, the boundary between the embryonic and extraembryonic regions is abnormal, and no morphologically distinct mesodermal layer forms. The lack of mesoderm induction observed in Smad4 null embryos is reminiscent of the phenotypes of embryos deficient in BMP-4 (Winnier *et al.*, 1995), BMPR-I (Mishina *et al.*, 1995), and ActRIB (Gu *et al.*, 1998). Visceral endoderm gene markers are absent or reduced in *Smad4-/-* embryos, but trophectoderm markers are expressed normally. *In vitro* culture of the mutant blastocysts reveals a decrease in cell proliferation (Yang *et al.*, 1998), which may be an explanation for the disturbed growth and development of the homozygous embryos. The surface of *Smad4-/-* embryoid bodies is covered with abnormal-appearing endoderm cells underlined by a thick extracellular matrix layer, whereas wild-type embryoid bodies are surrounded by well-developed visceral endoderm layer (Sirard *et al.*, 1998). Ex-

pression of visceral endoderm markers is either absent or greatly reduced at early stages of *in vitro* differentiation of *Smad4-/-* embryoid bodies, but the expression of certain mesodermal markers, including the T gene, recovers at later stages, suggesting that the lack of mesoderm is not an autonomous property of the *Smad4-/-* cells. To overcome the gastrulation block evident in *Smad4* deficient embryos, chimeras have been generated by aggregating *Smad4* null ES cells with wild-type tetraploid blastocysts (Sirard *et al.*, 1998). The majority of such chimeric embryos, in which visceral endoderm is derived exclusively from the wild-type cells, undergo gastrulation and at 8.5 days p.c. have somites. Some of these embryos develop to the head-fold stage, but they show anterior truncation, indicating that Smad4 expression in visceral endoderm is required not only for early gastrulation but also for patterning of the A-P axis.

b. Smad2

Like *Smad4*, *Smad2* is expressed ubiquitously throughout embryonic and extraembryonic tissues as early as 6 days p.c. (Nomura and Li, 1998; Waldrip *et al.,* 1998; Weinstein *et al.,* 1998). *Smad2-/-* embryos do not survive beyond 8.5 days p.c. (Nomura and Li, 1998; Weinstein *et al.*, 1998). At 6.0-6.5 days p.c., these embryos show growth retardation with visceral endoderm detached from the embryo and a disorganized boundary between embryonic and extraembryonic ectoderm. This appearance reflects a loss of proximo-distal asymmetry and subsequent lack of the A-P axis. Primitive streak is not formed, and neither embryonic nor extraembryonic mesoderm is induced. This phenotype is reminiscent of *Smad4* null embryos, but cell proliferation appears to be more attenuated in *Smad2-/-* embryos.

Some *Smad2* heterozygotes also exhibit defects in gastrulation; embryonic and extraembryonic mesoderm form, but these layers are unpatterned, indicating that Smad2 may function in both induction and patterning of mesoderm. In these animals a primitive streak forms, but does not extend to the distal tip. Some of the embryos develop outside of the yolk sac. Those embryos which survive beyond gastrulation exhibit craniofacial defects (Nomura and Li, 1998).

Trans-heterozygous *Smad2+/-*::*nodal+/-* animals have been created (Nomura and Li, 1998), based on the similarities of the null Smad2 and Nodal phenotypes. These compound heterozygotes show exacerbated defects compared to *Smad2+/-* embryos, both before and after gastrulation. In addition, the trans-heterozygotes exhibit cyclopia and defects in laterality not observed in either of the single heterozygotes. These results suggest that Smad2 is involved in the Nodal signaling pathway, possibly through type II Activin receptors.

Another Smad2 mutant in which a truncated form of Smad2 was used to generate homozygous embryos, exhibits a different phenotype, possibly because the targeted gene functions as a hypomorphic allele (Waldrip *et al.*, 1998). In these embryos, the extraembryonic tissues, including the ectoplacental cone, parietal endoderm, and visceral yolk sac, appear grossly normal (Waldrip *et al.,* 1998). However, the epiblast fails to develop into the embryo proper; instead, the entire epiblast assumes a proximal fate, giving rise only to extraembryonic mesoderm derivatives, including blood islands.

Chimera studies have been used to show that Smad2 expression in extraembryonic tissues (i.e., visceral endoderm) is required for normal development of the embryo proper (Waldrip *et al.,* 1998). Injection of wild type ES cells into Smad2 mutant blastocysts gives rise to chimeric embryos in which the visceral endoderm is composed exclusively of Smad2 mutant cells, while wild type cells contribute to the epiblast and its derivatives. These chimeric embryos closely resemble homozygous embryos produced by heterozygote matings, suggesting that *Smad2* expression in visceral endoderm is critical for specification of cell fate in the embryo proper. Together, these studies suggest that Smad2 is required for proper organization of the germ layer for gastrulation to proceed.

C. Other factors

1. Furin

This factor, encoded by the *fur* gene, is a protease implicated in the processing of proproteins such as insulin-like growth factor-I (Duguay *et al.*, 1997) and precursors of various TGF-β family members, including TGF-β1 (Dubois *et al.*, 1995), activin A (Roebroek *et al.*, 1993), Müllerian inhibiting substance (Nachtigal and Ingraham, 1996), BMP4 (Cui *et al.*, 1998), and Nodal (Roebroek *et al.*, 1998). At 7.5 days p.c. *fur* mRNA is expressed in extraembryonic endoderm and mesoderm, anterior visceral endoderm, and precardiac mesoderm, and later in the heart tube, lateral plate mesoderm, notochordal plate, and gut endoderm. *Fur-/-* embryos die by 10.5-11.5 days p.c. and exhibit ventral defects and abnormalities in fusion of the heart tube (Roebroek *et al.*, 1998). The foregut and midgut do not close, and the embryos do not turn (Roebroek *et al.*, 1998). In addition the yolk sac vasculature is disorganized, and the allantois does not attach to the chorion. While the *fur-/-* phenotype is reminiscent of that of *Gata4-/-* embryos (Kuo *et al.,* 1997; Molkentin *et al.*, 1997), GATA-4 expression appears normal in furin deficient mice. At the moment it is unclear whether these effects are due solely to the loss of furin in visceral endoderm. Nevertheless, these studies suggest that proteases, such as furin, may play a role in visceral endoderm signaling by regulating the accessibility of growth factors to specific regions of the embryo.

2. Hβ58

The *Hβ58* mouse line, produced by a random transgene insertion into chromosome 10, carries a recessive mutation that results in developmental abnormalities beginning at 7.5 days p.c. and embryonic death at approximately 9.5 days p.c. (Lee *et al.*, 1992). The gene disrupted by this insertional mutation encodes a 38 kDa protein homologous to the yeast secretory protein PEP8, a factor known to be essential for delivery of proteins to the vacuole in yeast (Bachhawat *et al.*, 1994). *Hβ58* mRNA is expressed in the embryonic and extraembryonic lineages of the post-implantation mouse. The visceral endoderm and ectoplacental cone express relatively high levels of *Hβ58* transcripts from 6.5 days p.c. onward. At 8.5 days p.c. all three germ layers of the embryo proper display a relatively low and spatially uniform level of *Hßβ58* mRNA. By 9.5 days p.c., abundant expression is seen in the visceral yolk sac and allantois. Mutant embryos display a severe reduction in the size of the embryonic ectoderm. While forming recognizable derivatives of all three germ layers, the embryos never exceed the size of a normal 8.5 day p.c. embryo. In addition, the allantois does not fuse with the chorion. The mutant embryos have a normal appearing visceral endoderm.

Based on the abundant expression of *Hβ58* mRNA in visceral endoderm it has been postulated that the developmental anomalies in the mutant embryos may reflect a defect in visceral endoderm function, although this hypothesis has not been tested directly.

3. Huntingtin

Huntington disease (HD) is an human autosomal dominant neurodegenerative disorder caused by expansion of a variable stretch CAG (glutamine) codons within a gene that encodes a large cytoplasmic protein, termed huntingtin (Jones *et al.*, 1997). The function of this protein is not well understood, but it localizes with the membranes of vesicles associated with microtubules. In mouse embryos the gene is expressed uniformly throughout all embryonic and extraembryonic tissues (Dragatsis *et al.*, 1998). Targeted mutagenesis of both alleles of the murine *HD* ortholog *(Hdh)* results in severe growth retardation and embryonic lethality (Duyao *et al.*, 1995; Nasir *et al.*, 1995; Zeitlin *et al.*, 1995). Although homozygous deficient embryos undergo gastrulation, they are disorganized in appearance and die by 10 days p.c.. In these mutants the morphology of the visceral endoderm is abnormal, and there is increased apoptosis in the distal epiblast (Zeitlin *et al.*, 1995), as in *Hnf4-/-* and *Gata6-/-* embryos. As shown by RT-PCR, there is normal expression of a variety of visceral endoderm markers, including *Hnf4* and *transferrin* (Dragatsis *et al.*, 1998). However, histochemical analysis suggests that iron transport by visceral endoderm may be abnormal (Dragatsis *et al.*, 1998).

Chimera studies have been used to show that huntingtin expression in extraembryonic tissues is required for normal development of the embryo proper (Dragatsis *et al.*, 1998). Injection of *Hdh-/-* ES cells into wild type blastocysts gives rise to chimeric embryos in which the visceral endoderm is composed exclusively of wild type cells, while Hdh-deficient cells contribute to the epiblast and its derivatives. These chimeric embryos are rescued from lethality. In contrast, when wild-type ES cells are injected into *Hdh* null blastocysts, the resultant chimeric embryos are morphologically indistinguishable from *Hdh-/-* embryos produced by heterozygote matings and die shortly after gastrulation. Collectively, these findings suggest that *Hdh* expression in visceral endoderm, trophectoderm, or extraembryonic ectoderm is critical for development of the embryo proper.

Factors normally not expressed in visceral endoderm which influence its function

1. Cripto

Cripto, also termed Cripto-1 (Cr-1), is a teratocarcinoma-derived growth factor belonging to the EGF-CGC gene family (Beddington, 1998; Ding *et al.*, 1998; Xu *et al.,* 1999). Cripto activates the ras/raf/MAP kinase pathway through an unknown receptor (Kannan *et al.*, 1997). This growth factor is not expressed in extraembryonic tissues, but is expressed uniformly in the pregastrulation epiblast (Ding *et al.*, 1998). Cripto expression becomes more pronounced in proximal epiblast before primitive streak formation, and during gastrulation expression shifts to the primitive streak, then rostral embryonic mesoderm. Mesoderm expression decreases at the early neural plate stage. By 8 days p.c., expression is confined to heart progenitors.

Cripto null mutants die before 10.5 days p.c. and lack primitive streak and embryonic mesoderm (Ding *et al.*, 1998; Xu *et al.*, 1999). These embryos contain extraembryonic mesoderm and a thickened layer of visceral endoderm, while ectoderm gives rise to distal ectopic folds of neural epithelium. The anterior character of this neuroepithelium is indicated by expression of the anterior and midbrain markers Otx-2, BF1, and En1. Anterior markers such as mCer-1 and Hex are expressed in the thickened distal visceral endoderm, while Hesx-1 is found throughout distal ectodermal layer. Thus, in *Cripto-/-* embryos anterior neuroectoderm is induced, but no posterior neuroectoderm forms, and the overall phenotype resembles a head without a trunk, the reverse of *Otx2-/-* or *Lim1-/-* mutant phenotypes. The *Cripto-/-* phenotype underscores the role of AVE as an anterior signaling center: misplaced AVE expresses gene products which in turn induce anterior differentiation in adjacent ectoderm. This phenotype also reinforces the notion of mutual signaling between visceral endoderm and epiblast. Certain mesodermal markers, such as GATA-4 and MEF2c, are expressed in *Cripto-/-* embryos, but late cardiac differentiation markers are not expressed (Xu *et al.*, 1999). In a similar fashion, differentiated cardiomyocytes are not observed in embryoid bodies derived from *Cripto-/-* ES cells (Xu *et al.*, 1998). Studies on fibroblasts isolated from mutant embryos indicate that changes in cell migration and adhesion to matrix contribute to the distorted appearance of *Cripto-/-* embryos (Xu *et al.*, 1999). The Cripto-deficient phenotype is not seen in chimeras generated by injecting *Cripto-/-* ES into wild type blastocysts, suggesting a paracrine effect for Cripto. It has been proposed that an early gradient of Cripto is required for correct orientation of anterior-posterior axis and proper establishment of both the head (AVE) and trunk (node) organizers by induction of proper cell movement of visceral endoderm and ectoderm (Beddington, 1998; Ding *et al.*, 1998).

2. Wnt8

In *Xenopus*, Wnt signaling molecules have been implicated in establishment of the Nieuwkoop signaling center, critical for induction of the Spemann organizer and axis specification (Kessler and Melton, 1998). In a similar fashion, the mouse *Wnt8* ortholog, normally expressed in the primitive streak but not in visceral endoderm, is believed to be a natural inducer of the primary signaling center responsible for axis formation (Bouillet *et al.*, 1996; Pöpperl *et al.*, 1998). In transgenic mice expressing chicken *Wnt8 (cWnt8)* under the control of a human β-actin promoter, there is ectopic expression of *cWnt8* in the visceral endoderm. These embryos exhibit incomplete axis duplication within a single amnion and anterior truncations (Pöpperl *et al.*, 1998). In addition, some of these embryos display a constriction at the extraembryonic-embryonic junction, while others lack a morphologically recognizable heart or foregut. Molecular studies demonstrate diminished expression of *Hesx1* in the anterior primitive endoderm. These results support the notion of the AVE as an early anterior organizer independent of the node/primitive streak function. Axis duplication may be incomplete because there is no prechordial plate to embellish anterior patterning. The *cWnt8* misexpression phenotype resembles that of mice bearing mutations of the *Fused* (Gluecksohn-Schoenheimer, 1949; Greenspan and O'Brien, 1986; Perry *et al.*, 1995) or *Lim1* genes (Shawlot and Behringer, 1995), suggesting antagonistic roles for these factors.

Developmental abnormalities associated with defects in imprinting and X-chromosome inactivation

1. Imprinting and non-random X-chromosome inactivation

There are several X-linked and imprinted autosomal genes expressed in visceral endoderm that have an effect on embryo development. In primitive endoderm and trophectoderm, there is selective inactivation of the paternal X-chromosome, while in other embryonic tissues X-chromosome inactivation is random (Takagi and Sasaki, 1975; Tan *et al.*, 1993). Improper X-chromosome inactivation results in developmental anomalies. Embryos carrying an extra copy of the maternal X-chromosome die because of a failure of ectoplacental cone and extraembryonic ectoderm development; these embryos also exhibit degenerating visceral endoderm and trophectoderm (Shao and Takagi, 1990; Takagi and Abe, 1990). The lethal phenotype of embryos disomic for the maternal X chromosome can be rescued by aggregation with wild type tetraploid embryos (Goto and Takagi, 1998), suggesting that failure of inactivation of one of the two X-chromosomes in primitive endoderm or trophectoderm derivatives is responsible for the phenotype.

Of interest, two transcription factor genes expressed in visceral endoderm have been mapped to the X-chromosome: a paired-like transcription activator *Esx-1* (Li *et al.,* 1997) and a Prd/Pax family related homeobox gene *Pem* (Sasaki *et al.*, 1991; Lin *et al.,* 1994; Lindsey and Wilkinson, 1996). Both genes are expressed also in adult testes, and Pem is expressed in ovaries and Kupffer cells, but the function of these genes is unknown. Homozygous null mutants of Pem do not exhibit abnormalities in visceral endoderm or gastrulation, suggesting that its function in visceral endoderm is dispensable (Pitman *et al.*, 1998).

X-chromosome inactivation requires the activity of the gene *Xist*, which is located at the X inactivation center and which is transcriptionally active only on inactive X chromosome (Panning *et al.,* 1997; Sheardown *et al.,* 1997). The product of this gene is a large, untranslated RNA. Inactivation of *Xist* by a deletion in the structural gene results in growth retardation and early embryonic death in female embryos carrying the mutation on their paternal X chromosome (Penny *et al.,* 1996; Marahrens *et al.,* 1997). Such embryos develop both visceral and parietal endoderm lineages, but yolk sac is either expanded or tightly adherent to the embryo and trophoblastic giant cells are abnormal. The mutant embryos either do not survive to gastrulation or are large and disorganized.

Among the autosomal genes that are imprinted in visceral endoderm are *H19*, *insulin-like growth factor-2 (Igf2)*, and the *insulin-like growth factor 2 receptor (Igf2r)* (Bartolomei *et al.*, 1993; Leighton *et al.,* 1996). *H19* is expressed from the maternal allele, and similar to *Xist*, it encodes the untranslated RNA which has been implicated in gene inactivation (Leighton *et al.*, 1995, 1996). The expression of this gene in extraembryonic lineages is temporally and spatially restricted during embryonic development (Poirier *et al.*, 1991). It is expressed in the late blastocyst and later on in the majority of extraembryonic lineages, including visceral endoderm. At 8.5 days p.c., *H19* expression is seen in embryonic organs (heart, intestine and liver among others), and it continues to be expressed in the adult heart. The expression of *H19* is necessary for inactivation of the maternal allele of *Igf2* (Leighton *et al.*, 1995). *Igf2* is expressed in trophectoderm derivatives and at 6.5-7 days p.c. its expression is observed transiently in the visceral endoderm in the lateral half of the epiblast and later on in the extraembryonic mesoderm. By 7.5 days p.c., *Igf2* is expressed in the yolk sac endoderm and anteroproximal embryonic mesoderm and its continuation in the yolk sac region. Visceral endoderm expression is no longer observed after 8 days p.c. (Lee *et al.*, 1990). In the embryo *Igf2* is expressed in the developing heart, head mesenchyme, and beneath the heart in the ventral and lateral walls of the foregut lining (Lee *et al.*, 1990). The circulating level of IGF2 is controlled by IGF2r which directs it to lysosomes (Dennis and Rifkin, 1991; Ludwig *et al.,* 1995). Loss of expression of *Igf2* results in growth retardation (Lau *et al.,* 1994). Mutation of the *H19* locus, like ablation of *Igf2r*, results in increased expression of *Igf2*, somatic overgrowth, and cardiomyocyte hypertrophy (Leighton *et al.*, 1995; Ripoche *et al.*, 1997). While *H19* null mice are viable, most *IGF2r* null mice die shortly after birth, and some of them die at the late embryonal stages probably from heart malfunction and edema (Lau *et al.,* 1994; Wang *et al.,* 1994; Ludwig *et al.*, 1996). Double *Igf2r*/*H19* mutants exhibit high IGF-2 levels and die during gestation beginning at day 12.5 (Eggenschwiler *et al.*, 1997).

2. Parthenogenetic embryos

The importance of imprinting in visceral endoderm function and embryonic development is underscored by studies on parthenogenones, embryos that contain only maternal DNA. In mouse chimeras derived from normal fertilized and parthenogenetic embryos, parthenogenetic cells contribute to the embryo proper and extraembryonic mesoderm, but not to extraembryonic endoderm or trophectoderm-derived tissues (Thomson and Solter, 1988). Parthenogenetic embryos derived from activation of diploid mouse oocytes develop to mid-gestation and show variable phenotypes, suggesting arrest at different developmental stages (Varmusa *et al.*, 1993; Sturm *et al.*, 1994). In the least affected embryos, which undergo gastrulation and develop proper A-P and D-V patterning, visceral endoderm shows a loss of characteristic apical vacuolization. The majority of these embryos show a reduction in forebrain structures that may reflect impairment of early signaling by AVE. In more disorganized embryos, ectoderm develops into a folded epithelium and visceral endoderm consists of a mixture of squamous and columnar cells. No primitive streak is formed and mesoderm, which migrates in disorganized fashion through the epiblast, becomes necrotic. The lack of axial patterning and embryonic/extraembryonic boundary in these embryos may result for the ectopic expression of early markers in disorganized visceral endoderm, similar to the phenotype of Crypto null embryos. Finally, in the most severe phenotypes, the whole epiblast is missing or reduced to a small core of ectodermal cells. These embryos develop extraembryonic tissue in the form of abundant parietal endoderm and giant cells encased in thick extracellular matrix with a complete lack of visceral endoderm (Sturm *et al.*, 1994). The different phenotypes described above may well represent disruption in particular functions of visceral endoderm, ranging from nutrient uptake to induction of gastrulation and axial patterning.

Similar to the tetraploid rescue experiments of null mutants described earlier, chimeric embryos produced by aggregating parthenogenones with wild type tetraploid embryos exhibit markedly improved development compared to non-chimeric parthenogenones (Spindle *et al.*, 1996). Day 12 chimeric em-

bryos have been identified in which the embryo proper is entirely derived parthenogenetically, while the visceral endoderm and trophectoderm are derived from wild type cells. These embryos show normal axial structures and organ formation. Still, parthenogenetic÷ tetraploid embryos fail to survive to late gestation because of defective chorioallantoic fusion (Spindle *et al.*, 1996).

Conclusions and future directions

Traditionally the visceral endoderm has been appreciated for its function in providing nutrition for the early embryo (Cross *et al.,* 1994; Copp, 1995). As highlighted in this review, it is now apparent that visceral endoderm has a far more complex role in early embryonic development, elaborating signals essential for normal embryogenesis. The portion of the visceral endoderm overlying the anterior epiblast functions as an organizer, helping to establish patterning in the embryo proper (Weinstein *et al.,* 1994; Thomas and Beddington, 1996; Ruiz i Altaba, 1998). The visceral endoderm itself is pre-patterned even before the onset of gastrulation (Thomas *et al.*, 1998), and regional differences in gene expression within the visceral endoderm layer influence development of the underlying embryo. Disruption of gene expression within select regions of the visceral endoderm, either through gene targeting (Table 1) or misexpression (Acampora *et al.*, 1998; Ding *et al.*, 1998; Pöpperl *et al.,* 1998), results in aberrant embryonic development. Some of these genes are expressed later in underlying epiblast derivatives. As exemplified by the phenotype of Oxt2 null mutant embryos in which the visceral endoderm function of Oxt2 was selectively rescued by expression of Otx1, the early expression of certain factors in AVE has an inductive function, while the subsequent expression in epiblast derivatives maintains or reinforces the induced structures (Acampora *et al.*, 1998; Suda *et al.*, 1999). The phenotypic effects associated with disruption of visceral endoderm include abnormalities in both A-P patterning [e.g., defects in rostral neuroectoderm with *Otx2* (Acampora *et al.*, 1995; Matsuo *et al.*, 1995; Ang H.L. *et al.,* 1996) and *Hesx1* (Thomas and Beddington, 1996)] and D-V patterning [e.g., defects in ventral morphogenesis with *Gata4* (Kuo *et al.*, 1997; Molkentin *et al.*, 1997; Narita *et al.*, 1997b) and *furin* (Roebroek *et al.*, 1998)]. In some instances, these patterning defects in the embryo proper are accompanied by abnormalities in gross morphogenic movements that affect the positioning of the yolk sac relative to the embryo [e.g., deficiencies in *Gata4* (Kuo *et al.*, 1997; Molkentin *et al.* 1997) and *HNF3β* (Farrington *et al.*, 1997)]. In all likelihood, signaling between visceral endoderm and subjacent ectoderm or mesoderm is not unidirectional (Farrington *et al.*, 1997; Beddington, 1998; Coucovanis and Martin, 1999). Changes in visceral endoderm morphology or gene expression that accompany an arrest in epiblast development (Table 1) may reflect, in part, disruption of reciprocal interactions between epiblast and endoderm.

Aspects of the organizer function of murine AVE are recapitulated in endodermal or mesendodermal cell layers found in other organisms (Bouwmeester and Leyns, 1997; Ruiz i Altaba, 1998). Rabbit embryos have thickened layers of AVE and anterior epiblast (Viebahn *et al.,* 1995). The primary hypoblast of chick embryos resembles the primitive endoderm of mouse embryos (Azar and Eyal-Giladi, 1981). This structure has a major role in both inducting the primitive streak and stabilizing the embryonic axis (Azar and Eyal-Giladi, 1979; Zoltewicz and Gerhart, 1997). Intriguingly, physical disruption of the chick hypoblast produces some of the same developmental abnormalities associated with physical or genetic disruption of the murine visceral endoderm. However, cross-species transplantation experiments indicate that chick hypoblast and mammalian visceral endoderm differ in their capacity to induce and pattern the vertebrate head. AVE from rabbit embryos, but not chick hypoblast, can induce formation of neuroectoderm and expression of anterior neural markers when transplanted into the area pellucida or area opaca of chick embryos (Knoetgen *et al.*, 1999). Thus, signals sufficient for head induction reside in the AVE of mammals, while in birds and amphibians these signals reside in prechordal mesendoderm. In *Xenopus*, the deep mesendodermal cells, which include progenitors of the anterior gut, exhibit functional similarities to the primitive endoderm of the mouse (Weinstein *et al.,* 1994; Belo *et al.*, 1997; Ruiz i Altaba, 1998) (Fig. 8). These mesendodermal cells express some of the same gene products critical for induction of ectoderm by murine visceral endoderm, such as Xcer (Bouwmeester *et al.*, 1996) and the *Xenopus* ortholog of Hesx-1 (Thomas and Beddington, 1996). The existence of an anterior organizer center is also evident from recent experiments in zebrafish. This center, named the anterior neural boundary (ANB), consists of a small population of anterior ectodermal cells, located between the yolk syncytial layer and the overlying periderm cells, which specify the most anterior region of the brain (Houart *et al.*, 1998). Transplantation experiments have shown that the yolk cell of the zebrafish is responsible for induction and patterning of the mesoderm (Mizuno *et al.*, 1996).

Many aspects of the differentiation and function of visceral endoderm are still poorly understood. Among these are the development cues responsible for initiating the vectoral movement of visceral endoderm cells and triggering regional gene expression in AVE as well as the role of embryonic and extraembryonic ectoderm in these processes. Future research should also shed light on other aspects of the role of the visceral endoderm in early development. Undoubtedly, the list of factors expressed in the AVE will expand by identifying novel molecules and by reexamining the early expression patterns of known factors. This may help define new organizer centers in migrating visceral endoderm. Recent studies on the inductive role of visceral endoderm in influencing posterior ectoderm cell fate (Belaoussoff *et al.*, 1998) suggest that regions of the visceral endoderm are functionally specialized early in gastrulation. Further exploration of the coincidental expression of factors in AVE and adjoining mesoderm, as is the case with GATA-4 and Mrg1, should help to establish the succession of factors and pathways participating in establishing anterior mesoderm fate. Tissue specific gene targeting may help to test the developmental consequences of selective disruption of gene expression in visceral endoderm. Forced expression of *Hesx1*, *Otx2*, *Lim-1*, *Hex* or *nodal* in other portions of the visceral endoderm might establish whether these molecules can alter specification of the neuroaxis, analogous to experiments in which Cwnt8 or Otx1 were misexpressed in visceral endoderm (Acampora *et al.*, 1998; Pöpperl *et al.*, 1998; Suda *et al.,* 1999). Given earlier studies showing that ectopic transplantation of the mouse node can induce a second neural axis (Beddington, 1994) and recent studies with chick embryos (Knoetgen *et al.*, 1999), transplanta-

tion experiments with different regions of visceral endoderm may be used to characterize other functions of visceral endoderm. Such studies should reinforce the expanding role of visceral endoderm as both a provider of nutrition and an organizer of development.

Summary

The murine visceral endoderm is an extraembryonic cell layer that appears prior to gastrulation and performs critical functions during embryogenesis. The traditional role ascribed to the visceral endoderm entails nutrient uptake and transport. Besides synthesizing a number of specialized proteins that facilitate uptake, digestion, and secretion of nutrients, the extraembryonic visceral endoderm coordinates blood cell differentiation and vessel formation in the adjoining mesoderm, thereby facilitating efficient exchange of nutrients and gases between the mother and embryo. Recent studies suggest that in addition to this nutrient exchange function the visceral endoderm overlying the egg cylinder stage embryo plays an active role in guiding early development. Cells in the anterior visceral endoderm function as an early organizer. Prior to formation of the primitive streak, these cells express specific gene products that specify the fate of underlying embryonic tissues. In this review we highlight recent investigations demonstrating this dual role for visceral endoderm as a provider of both nutrients and developmental cues for the early embryo.

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