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# The hypoblast (visceral endoderm): an evo-devo perspective

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## Summary
When amniotes appeared during evolution, embryos freed themselves from intracellular nutrition; development slowed, the mid-blastula transition was lost and maternal components became less important for polarity. Extra-embryonic tissues emerged to provide nutrition and other innovations. One such tissue, the hypoblast (visceral endoderm in mouse), acquired a role in fixing the body plan: it controls epiblast cell movements leading to primitive streak formation, generating bilateral symmetry. It also transiently induces expression of pre-neural markers in the epiblast, which also contributes to delay streak formation. After gastrulation, the hypoblast might protect prospective forebrain cells from caudalizing signals. These functions separate mesendodermal and neuroectodermal domains by protecting cells against being caught up in the movements of gastrulation.

**Key words: Chick, Endoderm, Evolution, Extra-embryonic tissues, Gastrulation, Mouse**

## Introduction
Long-term survival of all species is dependent on the fulfilment of nutritional needs during embryogenesis, which in higher metazoans is provided by cells derived from the embryonic endoderm. In amniotes, the subject of this review, not only does endoderm have important nutritional roles, but it also influences the development of other embryonic tissues. In most metazoans, the endoderm is merely the precursor of the lining of the adult gut, but amniotes evolved an additional, transient tissue: the primitive endoderm. Here we discuss the latter, which not only contributes to the establishment of the early embryonic body plan but also fixes it relative to the fetal membranes, thereby ensuring efficient exchange with the environment and thus, survival. In reptiles, birds and most mammals, this transient tissue is called ‘hypoblast’. In mice and some other experimental rodents, its equivalent is called ‘visceral endoderm’ (VE). Through regional compartmentalization, the hypoblast/VE positions the primitive streak, a dynamic structure that, via the movements of gastrulation, forms and distributes mesoderm and endoderm in an orderly manner throughout the embryo.

## Hypoblast/VE of amniotes: a yolk sac origin during evolution
Anamniotes (amphibians and fishes) reproduce by laying eggs that are released into their aquatic environment, sometimes as clutches attached to reeds or other objects. In this position, they are particularly vulnerable to predators, including adults of their own species. Perhaps for this reason, the embryos of many of these animals develop very quickly; their early cleavages occur without G1 or G2 phases and consist only of mitotic (M) and DNA synthetic (S) phases, and they rely upon maternal mRNAs and proteins until zygotic gene expression is activated, usually at around the 11th cell division (2024 cells). Rapid development and absence of growth imply that the embryonic axes or body plan must be set up quite quickly, within about 24 hours of fertilization, and by subdivision of the original mass of protoplasm, as seen in long germ band insects, such as *Drosophila*. Because of the absence of early growth, the fertilized egg of anamniotes contains all the nutrients necessary to sustain the embryo until it can feed itself, some time after hatching. In amphibians, nutrition is provided by intracellular yolk, and in teleost fish, it is a yolk mass that is internalized by the growing embryo (Trinkaus, 1984). In both fish and amphibians, the yolk ends up as gut contents within the hatched larva. At the time of hatching, the larva largely consists of a head-tail axis along which lie the spinal nervous system, striated axial muscles and a beating heart; the gut is not yet functional or even open to the outside, the brain is very rudimentary and there are few, if any, functional visceral organs. The larva is effectively a ‘swimming machine’ specialized to scatter away from the original egg clutch to avoid predation. Only then do the main organs start to develop.

Amniotes (reptiles, birds and mammals) evolved a profoundly different strategy for development (Arendt and Nübler-Jung, 1999). Although many of them are still oviparous, reproductive strategies involve laying fewer eggs and protecting them either by a calcareous shell, by burying them, or by providing more active maternal care. In most mammals and some reptiles, this was taken a step further with the appearance of viviparity, i.e. the embryo develops inside the mother’s body. Amniotes became adapted to life on land and evolved the amnion to provide a fluid environment in which the embryo floats and is protected from desiccation. They also perfected the yolk sac endoderm as an organ with which to acquire nutrients from outside the embryo itself, as well as to exchange oxygen. Although there is considerable variation between species, the yolk sac endoderm can be both a trophic tissue, taking up nutrients and other factors from the maternal environment in viviparous species (Beck, 1976), and probably also an inducing one, generating the yolk circulatory system and hematopoietic cells (Belaoussoff et al., 1998; Wilt, 1965); for a review on the evolution of fetal, or ‘extra-embryonic’, membranes see Ferner and Mess (Ferner and Mess, 2011) (see also below). This strategy allows more time for the embryo to develop, as well as enabling it to grow larger at early stages. The content of the fertilized egg cell became much less important as a nutritive source. Consequently, the zygotic genome is activated very soon after fertilization (Schultz, 2002) and even the earliest cell cycles possess G1 and G2 phases.

## Discovery of the primitive endoderm and hypoblast (VE)
In mammals, the existence of primitive endoderm and its association with both the extra-embryonic yolk sac and the embryo proper had been noted at the end of the 19th century. The primitive

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endoderm underlying the primitive ectoderm/epiblast became known as visceral endoderm, whereas that on the maternal side became parietal endoderm, as first recognized by Duval and Sobotta (Duval, 1891; Sobotta, 1911). Likewise, an early endoderm is described in the early chick embryological literature (Duval, 1889; Peter, 1938; Spratt and Haas, 1960; Vakaet, 1962).

However, many years passed before it became clear that the primitive endoderm is replaced by definitive endoderm, which mainly gives rise to the lining of the gut. The first clear experimental demonstration came from the careful experiments of Bellairs, who combined electron and light microscopy, cell marking and experimental embryology. Bellairs established that the deep layer of 'endoderm' present in chick embryos before the appearance of the primitive streak contributes to the extra-embryonic yolk sac stalk (Bellairs, 1953a; Bellairs, 1953b). Thus, the early chick embryo contains a transitory, extra-embryonic cell layer at its ventral surface. Its name, 'endoderm', was replaced by the term 'hypoblast', to distinguish it from definitive gut endoderm, which, as shown by Bellairs, is derived from the epiblast via ingression through the primitive streak, replacing the hypoblast. These observations were soon extended to reptiles by comparative morphological studies (Pasteels, 1957).

In mammals, comparable insights on the origin of definitive endoderm came mainly from several studies. These include the observations of Enders and colleagues in a number of different mammalian species (Enders et al., 1990; Enders et al., 1986; King and Enders, 1970), explant studies in rat (Levak-Svajger and Svajger, 1974; Svajger and Levak-Svajger, 1974), transplantation experiments by Gardner and colleagues in the mouse (Gardner, 1982; Gardner, 1984; Gardner, 1985; Gardner and Papaioannou, 1975; Gardner and Rossant, 1979), and eventually the clonal analyses of VE cells (Lawson et al., 1986; Lawson and Pedersen, 1987) and other important observations (Kadokawa et al., 1987; Lamers et al., 1987; for reviews, see Rivera-Perez et al., 2003; Rossant and Tam, 2009).

## Origin and cellular composition of the hypoblast/VE

### Development of the chick hypoblast

Relatively little is known about the origin of the hypoblast in the chick. At the time of egg laying, the central area (area pellucida) of the prospective embryo is a continuous layer of primitive ectoderm/epiblast underlain by 'islands', each containing 5-20 large yolky cells (Fig. 1A). The islands are thought to arise by poly-ingression, or cell shedding, from the very early epiblast (Fabian and Eyal-Giladi, 1981; Peter, 1938), but this has not been fully demonstrated. Gradually, the islands merge with each other, in a posterior-to-anterior direction, generating a complete layer beneath the area pellucida, the primary hypoblast (also called 'endophyll') (Stern, 1990a; Stern and Ireland, 1981; Vakaet, 1970) (Fig. 1B,C). This sequence of events is not identical in all birds; in turkey, for example, the hypoblast does not seem to form a layer in posterior-to-anterior sequence but instead forms either from the center or randomly (Bakst et al., 1997).

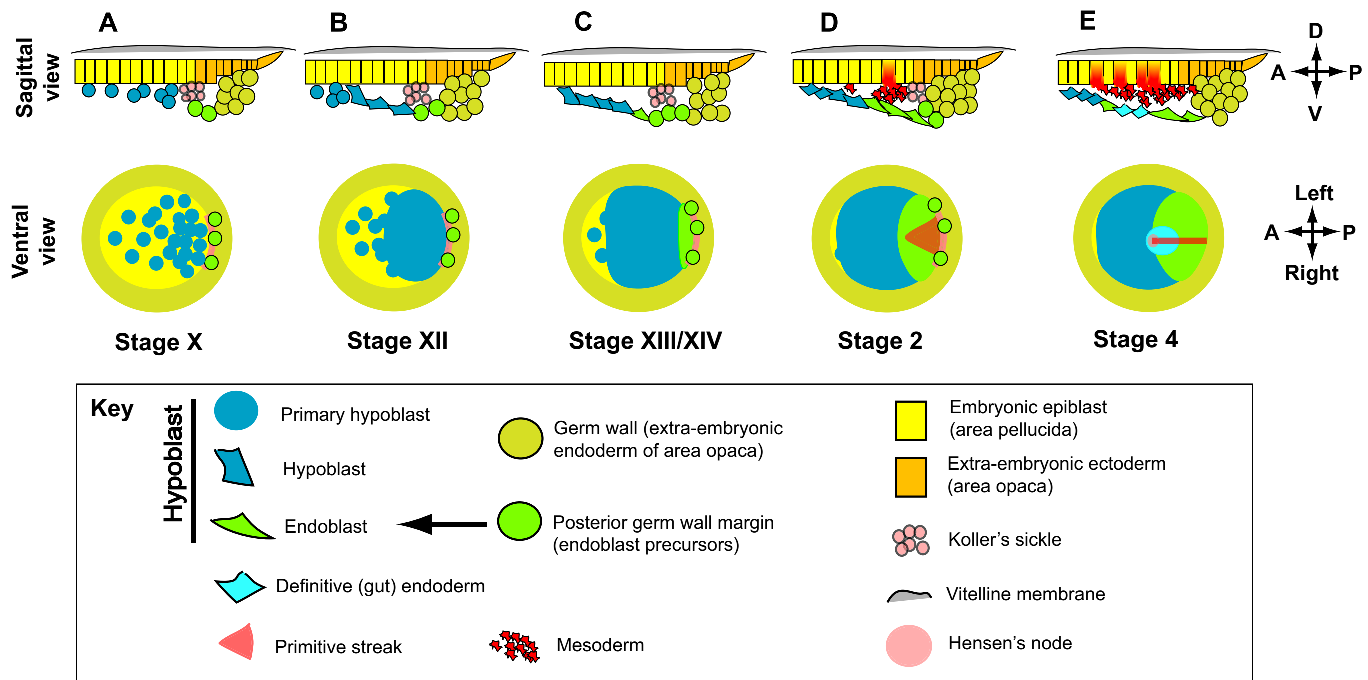


**Fig. 1. Development of the chick hypoblast and endoderm.** Top row: sections through the posterior part of the embryo; posterior (P) to the right, dorsal (D; epiblast) towards the top. Bottom row: ventral views of the whole embryo showing main anatomical features; posterior to the right, left side towards the top. Pre-primitive streak stages are shown in Roman numbers according to Eyal-Giladi and Kochav (Eyal-Giladi and Kochav, 1976). Primitive streak and later stages are shown in Arabic numerals according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). (**A**) Stage X (freshly laid embryo). The ventral surface is peppered with islands of primary hypoblast. Peripherally, there is a multilayered extra-embryonic endoderm (germ wall). Koller's sickle (pink) marks the posterior border of the area pellucida. (**B**) Stage XII. The islands of primary hypoblast have merged into a layer that covers the posterior half of the embryo. (**C**) Stage XIII/XIV. The primary hypoblast now makes a complete layer covering almost the entire area pellucida; the first (sickle) endoblast cells (green) appear posteriorly, derived from the posterior germ wall margin (green circles). (**D**) Stage 2. The endoblast continues to displace the primary hypoblast anteriorly. The primitive streak has appeared and cells now delaminate from the posterior epiblast to form mesoderm. (**E**) Stage 4. The primitive streak has now elongated to about two-thirds of the diameter of the area pellucida. Definitive (future gut) endoderm (light blue) has now inserted into the endodermal layer.

Surrounding the area pellucida is the area opaca, an extra-embryonic region underlain by layers of even larger, yolky cells called the germ wall (Fig. 1), the edge of which appears to generate flatter cells that spread a short distance (one to two cells) into the area pellucida to make a peripheral ring, the junctional endoblast (Low and McClugage, 1993; Stern and Ireland, 1981; Vakaet, 1962; Vakaet, 1970). Posteriorly, the encroachment of these cells is much greater and progressive, and generates a layer, called sickle endoblast (or just endoblast), that displaces the original hypoblast in an anterior direction (Stern, 1990a; Vakaet, 1970) (Fig. 1C,D). The term 'sickle' is a reference to Koller's sickle, a crescent-shaped ridge of middle layer cells just beneath the epiblast at the border between area pellucida and the posterior marginal zone (Fig. 1).

At almost exactly the same time as the endoblast enters the area pellucida, the primitive streak appears, quite quickly, in the overlying epiblast (Fig. 1D), suggesting a connection between the two events (Bertocchini and Stern, 2002). Collectively, the primary hypoblast, junctional endoblast and sickle-derived endoblast form a continuous layer which is also generally known as the hypoblast (occasionally and more appropriately, 'lower layer'). These components of the hypoblast layer can be distinguished morphologically (Stern and Ireland, 1981) as well as by expression of a number of molecular markers, some of which will be mentioned below together with their mouse counterparts.

### Development of the mouse VE

In mouse, the VE segregates from the primitive endoderm of the implanting blastocyst [embryonic day (E) 4.5-5.5] (Fig. 2A) as the latter elongates to become an egg cylinder (Fig. 2B). Following molecular observations by Martin and colleagues (Rosenquist and Martin, 1995) and morphological, behavioral and molecular observations by Beddington and colleagues (Thomas and Beddington, 1996), the embryonic VE (EVE) was discovered to contain a morphologically distinct domain at the distal tip of the mouse egg cylinder (~ E5.5) called 'distal visceral endoderm' (DVE) (Fig. 2B).

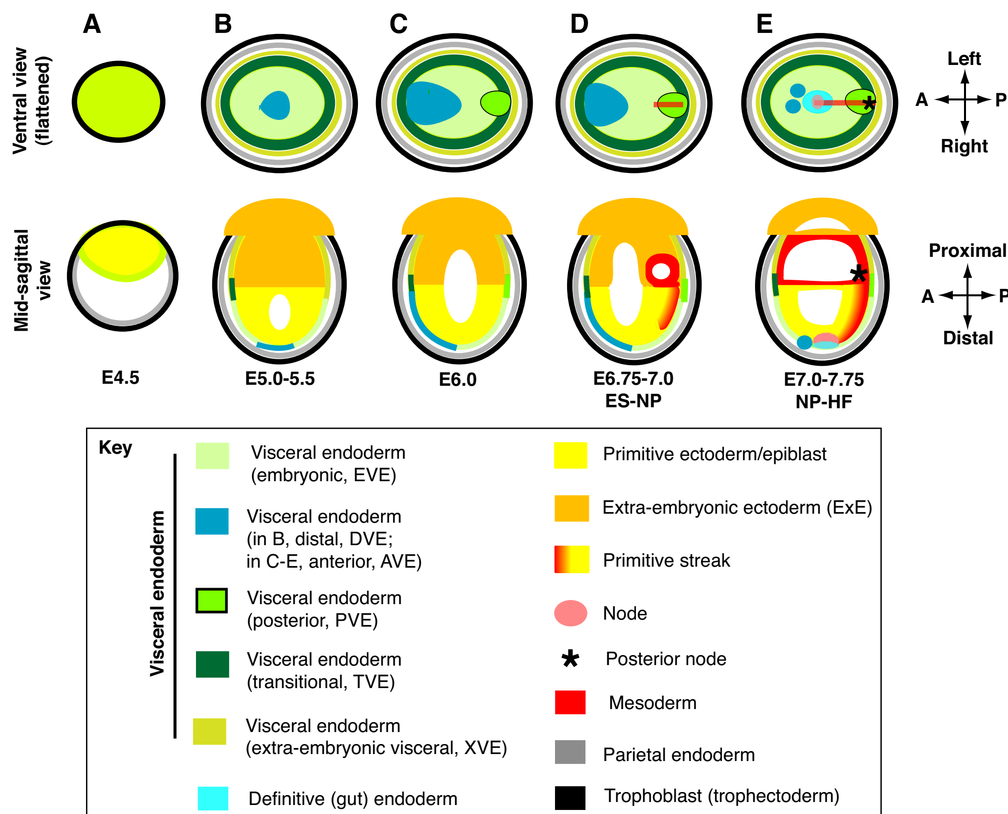


**Fig. 2. The visceral endoderm (VE) during early mouse development (~E4.5-7.75, pre-streak to head fold stages).** Top row: views from the visceral endodermal side (parietal endoderm and associated trophoblast, which would obliterate this view, are shown only on the periphery of each figure); posterior (P) to the right, left side to the top. Bottom row: sagittal sections through the conceptus; posterior to the right, maternal (implantation or proximal) side is towards the top. (**A**) E4.5. The implanting blastocyst contains epiblast (yellow), VE (green) and parietal endoderm (gray). Trophoblast (black) is indicated. (**B**) At ~E5.0-5.5, the distal visceral endoderm (DVE) forms a group of cells within the embryonic visceral endoderm (EVE). (**C**) By ~E6.0, the DVE has spread to the anterior embryonic/extra-embryonic border, where it is now called anterior visceral endoderm (AVE). At around the same time, a molecularly distinct posterior visceral endoderm (PVE) emerges. (**D**) E6.75-7.0 [early streak (ES)-neural plate (NP) stage (Downs and Davies, 1993)]. By ~E6.75, the primitive streak has appeared in the posterior axial midline of the epiblast, and extends anteriorly. (**E**) By E7.0 [NP-headfold (HF) stages], the anterior end of the primitive streak has condensed into the node (pink) and posteriorly it extends into the extra-embryonic region where, perhaps together with transitional visceral endoderm (TVE) and PVE, it condenses into the 'allantoic core domain' (ACD) or posterior node (Downs et al., 2009). By this time, the AVE seems to disappear into the anterior extra-embryonic visceral endoderm (XVE), though some AVE cells (dark blue) might remain interspersed with definitive (gut) endoderm (Kwon et al., 2008). With the exception of the DVE, which is morphologically distinct from the EVE, the EVE is characterized by its flat squamous cells, largely without microvilli or apical vacuoles. TVE is cuboidal containing some microvilli and vacuoles, and XVE is columnar with many microvilli and apical vacuoles (Bonnevie, 1950; Enders et al., 1978; Hogan and Tilly, 1981; Solter et al., 1970).

The DVE acquires its properties by induction from nearby cells. Although it has not yet been established that this induction is truly instructive, and the signaling and responding cells have not yet been definitively identified, it is clear that regional signals from extra-embryonic ectoderm (ExE), which borders the epiblast, are required (Richardson et al., 2006; Rodriguez et al., 2005) and that positioning the DVE requires the Nodal/BMP/Smad signaling pathways (Camus et al., 2006; Chazaud and Rossant, 2006; Chen et al., 2006; Mesnard et al., 2006; Yamamoto et al., 2009). Subsequently, DVE cells migrate anteriorly (Fig. 2B,C) over the epiblast, stopping when the leading edge reaches the embryonic/extra-embryonic junction, where epiblast is juxtaposed to ExE (Thomas and Beddington, 1996) (Fig. 2C). Here, the DVE cells become known as 'anterior visceral endoderm' (AVE) (~E6.0) (Fig. 2C). Thus, the DVE/AVE undergoes anterior movements equivalent to the chick primary hypoblast and both tissues express similar markers, as summarized later.

Although not as well studied as the AVE, there appears to be a separate posterior visceral endoderm (PVE) cell population at the posterior edge of the embryonic territory, which seems to be a behaviorally and molecularly distinct type of EVE (Fig. 2C). The PVE is apparent during early streak stages and is identified by the expression of Mixl1 and Wnt3a (Hart et al., 2002; Pearce and Evans, 1999; Pfister et al., 2011; Rivera-Perez and Magnuson, 2005; Robb et al., 2000). During early streak stages, PVE forms part of the EVE population associated with the posterior primitive streak (Fig. 2D,E) that is not displaced, at least by the early neural plate (E8.0) stage, by endoderm recruited from the epiblast (Tam and Beddington, 1992). At neural plate stages, the PVE might be part of the transitional visceral endoderm (TVE) that overlies the extra-embryonic/embryonic junction (Bonnevie, 1950), where it might establish a posterior node and the allantois (Downs, 2009; Downs et al., 2009) (Fig. 2E).

In the chick, the (sickle) endoblast persists to the late primitive streak stage (stage 4). As in the mouse (see below), Wnt3a is expressed in the posterior region at this time, although it cannot be detected by in situ hybridization at earlier stages. It therefore seems likely that the chick endoblast is equivalent to the PVE, perhaps including at least part of the non-anterior VE.

## Gene expression in the hypoblast and VE

The first marker to identify a subregion of mouse EVE (Fig. 2) was antigen VE-1 (Rosenquist and Martin, 1995), providing clues that the EVE is molecularly regionalized. Then, a number of gene products, mainly transcription factors that are also found in other tissues, such as the node and/or anterior neural plate, started to be found in both chick primary hypoblast and mouse EVE, including the homeobox genes *Otx2* (Acampora et al., 2009; Albazerchi et al., 2007; Bally-Cuif et al., 1995; Kurokawa et al., 2010; Perea-Gomez et al., 2001), *Hex* (Yatskievych et al., 1999) and *Hesx1/Rpx* (Chou et al., 2006; Thomas and Beddington, 1996), and others encoding transcription factors of the GATA and SOX families (Artus et al., 2011).

A number of secreted factors, including Fgf8, retinoic acid (RA) and several inhibitors of other secreted factors, such as Cerberus and Dickkopf 1 (Dkk1), are also expressed in both chick hypoblast and mouse AVE (Albazerchi and Stern, 2007; Bally-Cuif et al., 1995; Belo et al., 1997; Bertocchini and Stern, 2002; Bertocchini and Stern, 2008; Knezevic and Mackem, 2001; Knezevic et al., 1995; Matsui et al., 2008; Perea-Gomez et al., 2002). However, there are also some differences; for example, *Lefty1* is expressed in the mouse AVE but an orthologous gene does not seem to exist in the chick. Recently, several groups have conducted molecular screens to discover markers specific to hypoblast/AVE, which, although they added many more gene products to this list, have not yet revealed any that are specifically expressed in the chick endoblast or in the mouse (non-anterior) VE (Bertocchini and Stern, 2008; Gonçalves et al., 2011).

Recent genetic lineage mapping (Takaoka et al., 2011) has challenged the simple progenitor-descendant relationship between DVE and AVE. It was proposed that *Lefty1*-expressing epiblast cells contribute to the DVE and that the AVE is not derived from these but rather from *Lefty1*-negative EVE cells that move to the distal tip. However, the DVE is required for anterior migration, with future anterior-posterior (A-P) polarity of the embryo being determined by the *Lefty1*$^{+}$ cells in epiblast. This model is consistent with reports that the AVE arises partly from de novo gene expression in new cells and partly by early cells (Torres-Padilla et al., 2007). As in birds, there are differences in this topology and sequence of events among different mammals, including the rabbit (Viebahn et al., 1995), primates (Enders et al., 1990; Enders et al., 1986), pig (Hassoun et al., 2009) and monotremes (Kress and Selwood, 2006; Selwood and Johnson, 2006).

## Movements of the hypoblast/VE

It is now clear both in chick (Gräper, 1929; Peter, 1938; Spratt and Haas, 1960; Vakaet, 1970; Wetzel, 1929) and in mouse (Thomas and Beddington, 1996) that the hypoblast/DVE (prospective AVE) moves anteriorly just before primitive streak formation. However, the mechanisms that control this translocation, and especially those that determine directionality of movement, are only just starting to be elucidated, and so far only in the mouse. Epiblast cell proliferation is required for AVE migration (Stuckey et al., 2011a); perhaps this relates to the change in shape of the mouse egg cylinder that accompanies AVE migration (Perea-Gomez et al., 2004; Rivera-Perez et al., 2003; Rossant and Tam, 2009), which might accentuate the movement of the AVE. Although it was originally thought that the AVE moves anteriorly as a coherent group of cells, it is now becoming apparent that these cells might move within, or among, the surrounding EVE (Stuckey et al., 2011a; Stuckey et al., 2011b; Takaoka et al., 2011; Trichas et al., 2011), a process that would require sorting as well as guidance mechanisms.

There is evidence for the involvement of intercellular signals, such as angiomotin (Shimono and Behringer, 2003), Nodal and Wnt in DVE movement; Nodal- and Cripto-null embryos, for example, show defects in this migratory event. Specifically, Nodal has been proposed to drive DVE migration by localizing proliferation within the EVE, whereas the Nodal antagonists Lefty and Cerl provide guidance cues anteriorly (Yamamoto et al., 2004). In a similar model, canonical Wnt and Dkk1, a Wnt antagonist, were proposed to act as repulsive and attractive guidance cues, respectively (Kimura-Yoshida et al., 2005). Thus, prospective AVE cells might follow decreasing gradients of Nodal and canonical Wnt activity. The non-canonical, planar cell polarity (PCP) pathway is also involved; it has been proposed that EVE cells drive anterior migration of the DVE through the Wnt-PCP pathway, which in turn appears to be modulated by Nodal (Trichas et al., 2011). Rac1 is also essential for this migration (Migeotte et al., 2010), perhaps as a target of PCP.

## Fate of the hypoblast/VE

The gut endoderm in both mammals (Tam and Beddington, 1987; Wilson and Beddington, 1995) and birds (Bellairs, 1953a; Bellairs, 1953b; Kimura et al., 2006; Psychoyos and Stern, 1996; Selleck and

Stern, 1991) arises mainly from the anterior primitive streak and node. However, there might also be a contribution from the posterior primitive streak for the cloacal region (Hassoun et al., 2010) and within the allantois (Mikedis and Downs, 2012). In addition, recent findings have raised the possibility that remnants of the AVE might remain in the final endodermal layer and contribute to the gut (Kwon et al., 2008) (Fig. 2E). This is consistent with observations in chick that some hypoblast cells are retained under the primitive streak (Azar and Eyal-Giladi, 1983) and that ingressing prospective gut endoderm cells migrate a long way within the mesodermal layer before inserting into the deep layer (Kimura et al., 2006), which might provide a mechanical explanation for the retention of hypoblast cells. 'Invasion' of primary hypoblast by definitive gut endoderm might occur because of higher adhesion/cohesion of the latter (Ireland and Stern, 1982; Sanders et al., 1978).

By E7.0 in mouse, the AVE cell population appears to have dispersed along the boundary of embryonic and extra-embryonic regions and to the anterior half of the extra-embryonic yolk sac (Lawson and Pedersen, 1987; Rivera-Perez et al., 2003; Shimono and Behringer, 2003; Tam et al., 2007). In chick, the descendants of the primary hypoblast become confined to an anterior region known as the 'germinal crescent' because it also contains the primordial germ cells (see below). Fate-mapping studies of the VE at different stages of gastrulation show that cells lying between the AVE and the PVE (i.e. much of the remaining EVE) (Fig. 2B-E), will contribute to yolk sac visceral endoderm (Lawson and Pedersen, 1987; Tam et al., 2004; Tam et al., 2007). Thus, the EVE as a whole resembles the chick hypoblast layer, which contributes to the yolk sac stalk (Bellairs, 1986).

## Functions of the hypoblast/VE

The preceding sections surveyed the embryonic origin, movements and fate of the hypoblast/AVE and proposed that this tissue is likely to be an amniote innovation during evolution. Here, we briefly review the various functions acquired by this novel tissue.

### Positioning the site of gastrulation (primitive streak)

The greater amount of growth that amniote embryos undergo at early stages requires not only a source of nutrition for the embryo but also a mechanism that allows this rapid growth to occur. This can involve a growth zone (blastema) in which cell division is concentrated and from which cells can be distributed to other parts of the embryo. To achieve this, amniote embryos relocated the site of gastrulation from the periphery of the embryo (where the blastopore of amphibians and the embryonic shield of teleosts are located) to the midline, generating the primitive streak. The primitive streak is both a passageway for epiblast cells to enter the deeper layers of the embryo and a growth zone, as it functions for a very long time. Despite being at the midline of the embryo, the fates of cells residing within the axis of the primitive streak do not correspond to the A-P axis of the embryo, but rather to the axial-lateral dimension. Specifically, the anterior part of the primitive streak (including Hensen's node) contains notochord and somite precursors, followed by heart, intermediate mesoderm, lateral plate and extra-embryonic mesoderm at the 'posterior' end (Kinder et al., 1999; Psychoyos and Stern, 1996; Selleck and Stern, 1991; Tam and Tan, 1992). The nervous system caudal to the hindbrain develops from small regions of epiblast next to the node (Brown and Storey, 2000; Stern et al., 2006). In this way, amniotes have adopted a mode of head-tail axis formation relying on sequential caudal addition more similar to that of short germ band insects (e.g. grasshoppers) and annelids, than that of anamniotes (Stern, 1990b).

Recent data have suggested that the posterior end of the streak extends into what has hitherto been regarded as extra-embryonic territory, the allantois, where, in the mouse, it might establish a posterior node-like structure (Downs et al., 2009), first suggested by Mulnard (Mulnard, 1956; reviewed by Downs, 2009). A similar node-like structure, with mesoderm-inducing properties, has been described in chick (Vakaet, 1973).

Many observations made in the last fifteen years suggest that repositioning of the site of gastrulation involves two key steps: new cell movements in the epiblast to drive this relocation, and a concomitant delay in the onset of primitive streak formation while the movements take place. Evidence points to the hypoblast/AVE as the tissue responsible for controlling both of these steps.

#### Regulation of cell movements in the epiblast

After rotating the deep layer (hypoblast) in chick embryos before the start of gastrulation, Waddington observed that the orientation of the primitive streak followed that of the rotated layer rather than that of the epiblast from which the streak arises (Waddington, 1932; Waddington, 1933). Waddington proposed that the rotated hypoblast induces formation of the primitive streak (Azar and Eyal-Giladi, 1981; Eyal-Giladi and Wolk, 1970; Waddington, 1933). However, later experiments suggested that what Waddington was really observing was an effect of the hypoblast on cell movements rather than on epiblast cell fate. Labeling specific regions of the epiblast followed by hypoblast rotation revealed that the primitive streak, whose orientation now followed that of the hypoblast, contained the cells that would normally have contributed to the original axis, but in a new position (Foley et al., 2000). In other words, rotation of the hypoblast alters the pattern of cell movements in the overlying epiblast, which causes the primitive streak to bend.

Further insight into the nature of epiblast movements and the interaction between epiblast and hypoblast came from more recent experiments, in which the forces driving the 'Polonaise' (double-whorl) global movements of pre-primitive streak-stage chick embryos were examined (Voiculescu et al., 2007). It was found that mediolateral cell intercalation at the posterior edge of the embryonic epiblast causes narrowing and midline elongation (a convergence-extension movement) of this domain of cells, which contains the bulk of primitive streak precursors. This intercalation was shown to require the Wnt-PCP pathway in the epiblast, including the expression of Prickle, Flamingo and VanGogh-like2 (Voiculescu et al., 2007). Hypoblast rotation induces ectopic expression of these genes and generates a new center for the Polonaise movements. Hypoblast rotation can be mimicked by implanting a bead of Fgf8 (a factor normally expressed in the hypoblast by this stage) (Voiculescu et al., 2007). These results suggest that fibroblast growth factor (FGF) from the hypoblast induces PCP genes in the adjacent epiblast, which in turn causes cells to intercalate mediolaterally and extend the prospective streak domain to lie along the midline of the embryo, bringing prospective organizer cells (anterior primitive streak and node) to the middle of the blastoderm. This explains the results of Waddington's original hypoblast rotation experiment. Although to date this has not been demonstrated in the mouse, fate maps and other observations suggest that the mouse AVE and the adjacent epiblast might indeed move anteriorly in concert before primitive streak formation (Thomas and Beddington, 1996; for reviews, see Rivera-Perez et al., 2003; Rossant and Tam, 2009). Thus, the hypoblast directs mediolateral intercalation of epiblast cells at the posterior edge of the embryonic epiblast, which contributes to place the primitive streak at the midline.

DEVELOPMENT

### Inhibition (delay) of primitive streak formation

Two other observations contributed to refine this view of hypoblast-directed epiblast movements. In chick, it was observed that removal of the hypoblast caused multiple, ectopic primitive streaks to appear, and evidence was provided for an involvement of the Nodal antagonist Cerberus, produced by the hypoblast (Bertocchini and Stern, 2002). In the chick, Nodal is expressed in the adjacent epiblast, the same region that undergoes mediolateral intercalation, contains the streak precursors and is required for primitive streak formation (Bertocchini and Stern, 2002; Skromne and Stern, 2002). It was observed that the primitive streak starts to form at precisely the same time as the endoblast starts to underlie the streak-forming area, effectively removing the hypoblast from this domain; because the endoblast does not express Cerberus, this suggested that a function of the hypoblast might be to delay primitive streak formation until the time of endoblast formation.

At the same time, it was shown that mice carrying mutations in two Nodal antagonists, Cerberus and Lefty1, both of which are expressed in the AVE, develop with multiple ectopic primitive streaks (Perea-Gomez et al., 2002). There are a few differences in the precise pattern of expression of these components between mouse and chick. For example, the mouse *Lefty1* does not have a direct ortholog in the chick (the gene labeled *Lefty-1* in chick has properties more similar to mouse *Lefty2*, which is expressed later), and Cerberus is the only Nodal antagonist known to be expressed in the hypoblast (Bertocchini and Stern, 2002). *Nodal* mRNA is more ubiquitously expressed in mouse than in the chick (Brennan et al., 2001; Skromne and Stern, 2002). However, similar interactions between Nodal, Cerberus and Lefty appear to occur in the rabbit (Idkowiak et al., 2004); thus, it seems likely that there is a common mechanism by which the hypoblast/AVE represses primitive streak formation until the appropriate stage in development. In addition, Wnt3a secretion by the PVE (or endoblast in the chick) might cooperate with Nodal to induce the appearance of the streak at the appropriate time (Rivera-Perez and Magnuson, 2005; Robb et al., 2000).

More recent findings suggest that the inhibition of primitive streak formation by the hypoblast might involve additional mechanisms, perhaps including inhibition of the epithelial-mesenchymal transition (EMT). The AVE was proposed to act by depositing extracellular matrix components that act as a ‘morphogenetic barrier’ to inhibit EMT, and involving Flrt3 (Egea et al., 2008). This might also be correlated with the observation that the hypoblast deposits basement membrane components onto the inner epiblast surface at this stage in the chick (Harrisson et al., 1985).

Together, these observations provide evidence for an important role of the hypoblast/AVE in positioning the primitive streak at the midline of the amniote embryo. This is achieved by a combination of a temporary block to primitive streak formation by several mechanisms, including Nodal inhibition and blocking the EMT, until the precursor cells are relocated to the midline through a process of cell intercalation that involves the Wnt-PCP pathway.

## Separating head from trunk/tail, and ‘pre-neural’ induction

The relocation of the site of gastrulation from the blastopore/shield of amphibians and fish to the midline of the amniote embryo in the form of the primitive streak effectively places a ‘plug-hole’ in the center of the embryo. Unless this hole is plugged, much of the epiblast could be lost to deeper layers by ingression, with disastrous consequences to development of the embryo. This reorganization therefore requires a mechanism to prevent some cells from ingressing so that they can give rise to surface ectoderm and the nervous system. An early function of the hypoblast/AVE, reviewed above, is to delay primitive streak formation; protecting a specific portion of the epiblast against ingression seems like a logical continuation of this function. As discussed above, the hypoblast/AVE moves forward just as the primitive streak starts to form, ending up in a position adjacent to the future head ectoderm, including the cephalic neural plate.

### What the hypoblast/AVE does not do: head induction

The first study to suggest a causal relationship between the hypoblast and cephalic neural development was based on trans-filter recombinations of hypoblast and epiblast in the chick (Eyal-Giladi and Wolk, 1970), suggesting that soluble factors secreted by the hypoblast have prosencephalic (forebrain)-inducing properties. The authors proposed: “The primary hypoblast, by virtue of its prosencephalic inducing power, can be compared with the presumptive pharyngeal endoderm of the amphibian embryo” (Eyal-Giladi and Wolk, 1970). But this observation was essentially forgotten until a quarter of a century later when Beddington observed in mouse that the movement of the AVE anteriorly mirrors epiblast movements, that the final position of the former is adjacent to the prospective forebrain, and that removal of the AVE leads to changes in gene expression in the future forebrain (Thomas and Beddington, 1996). Others interpreted the results of this experiment as implying that the AVE can induce the forebrain directly from epiblast, acting as a ‘head organizer’. Because misexpression of Wnt antagonists generates ectopic head structures in *Xenopus* (Glinka et al., 1997), it was tempting to suggest that Cerberus and/or Dkk1 expression in the mouse AVE might be directly responsible for something similar (Belo et al., 1997; del Barco Barrantes et al., 2003; Glinka et al., 1998). However, the AVE by itself does not induce either neural or stable forebrain markers in mouse (Tam and Steiner, 1999), nor does the hypoblast induce the expression of neural markers in chick (Albazerchi and Stern, 2007; Foley et al., 2000; Foley et al., 1997). The latter does induce expression of a number of genes in the epiblast (Sox3, Otx2, Not1/Not2 and Cyp26A1), several of which are also expressed at later stages in the nervous system or the forebrain (Albazerchi and Stern, 2007; Bally-Cuif et al., 1995; Knezevic and Mackem, 2001; Knezevic et al., 1995). However, this induction, which is due to multiple signals that probably include FGF, RA, Wnt antagonists and Cerberus (Albazerchi and Stern, 2007; Bally-Cuif et al., 1995; Knezevic and Mackem, 2001; Knezevic et al., 1995), is only transient and is certainly not sufficient to impart either neural or forebrain identity to epiblast cells outside the region fated to form this tissue (Albazerchi and Stern, 2007; Foley et al., 2000; Foley et al., 1997; Stern, 2001; Stern et al., 2006).

There is no question, however, that both the mouse AVE and the chick hypoblast are required for normal development of the head, including the brain. This is supported, for example, by numerous genetic studies in which the AVE fails to form or to migrate properly, in which head development is perturbed (Acampora et al., 2009; Clements et al., 2011; Martinez-Barbera et al., 2000; Martinez Barbera et al., 2000; Rivera-Perez et al., 2003; Rossant and Tam, 2009). However, the requirement for the AVE in brain formation is not due to direct induction of either forebrain or neural character on naive ectoderm by the hypoblast/AVE. The early and transient expression of specific proteins has been proposed to represent a ‘pre-neural/pre-forebrain’ state, which requires

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stabilization by signals from other tissues, including the organizer (anterior primitive streak and Hensen's node), for cells to acquire either neural or forebrain identity (Pinho et al., 2011; Stern, 2001; Stern et al., 2006; Streit et al., 2000). However, it is puzzling that mouse embryos lacking functional Nodal signaling in the AVE, and the AVE itself, show generalized pre-neural gene expression, rather than lack thereof (Camus et al., 2006). This is likely to be due to a combination of roles for Nodal both in AVE development and in adjacent epiblast (Brennan et al., 2001).

### Separation of head from trunk/tail

If the influence of the hypoblast/AVE in head and brain development is not by direct instructive neural or forebrain induction, how does it work? It seems most likely that this is due to a continuation of the anti-primitive streak-forming activity seen at early stages. This might involve antagonists of Nodal and Wnt as well as perhaps an anti-EMT, as discussed above. Anti-Wnt signals, such as Dkk, might also contribute to continued protection against caudalization of the early nervous system, as there is considerable evidence that Wnt acts as a strong neural caudalizing signal in all vertebrates (Glinka et al., 1998; Wilson and Houart, 2004). At later stages, the protection against neural caudalization might be reinforced by the anterior definitive endoderm, because repeated ablation of this tissue leads to forebrain defects (Withington et al., 2001). This might be a conserved ancestral role of the anterior gut endoderm of lower vertebrates (Pera and De Robertis, 2000; Schneider and Mercola, 1999; Smithers and Jones, 2002).

Therefore, the hypoblast/AVE is involved in head and forebrain development through at least three sequential 'protective' events (Foley et al., 2000; Kimura et al., 2000): the hypoblast/AVE protects the anterior region of the epiblast first against premature primitive streak formation, then against being caught up in ingression once the primitive streak has formed, and finally against caudalization of the recently induced cranial nervous system by inhibiting Wnt. These functions are distinct (and occur at different times), though their combined net effect ensures that the head ectoderm can form properly. We propose that all are consequences of the evolutionary innovation of moving the site of gastrulation to the midline rather than an 'A-P patterning' event, which occurs quite differently in amniotes and anamniotes.

## Other functions of the hypoblast/VE

The functions discussed above – positioning the site of gastrulation, protecting future neuroectodermal and head epidermis from ingression, protection against caudalizing signals, and transient induction of 'pre-neural' genes – are likely to represent the major roles of the hypoblast/VE in development. However, there is evidence for several additional functions of the VE, at least in a subset of amniotes.

### Trophic functions and relations to the circulatory system

As discussed above (see also Arendt and Nübler-Jung, 1999), a major function of the yolk sac, in both anamniotes and amniotes, is to provide nutrition to the embryo in one form or another. In the chick, the hypoblast is made up of large cells containing yolky and glycogen inclusions (Bellairs, 1964; Wolk et al., 1982). Apolipoproteins are expressed in both chick hypoblast and mouse AVE (Bertocchini and Stern, 2008; Shi and Heath, 1984). These proteins have been suggested to be involved in yolk and lipoprotein metabolism (Farese et al., 1996); therefore, their expression in the mouse underscores the common evolutionary origin of these two tissues in birds and mammals.

Egg-laying amniote embryos are closely juxtaposed to a very large mass of yolk that is digested by yolk endoderm. In response to the need for oxygen and waste storage, the amniote yolk endoderm took on the new property of inducing the mesoderm to produce a specialized circulatory system, complete with hemoglobin-transporting erythrocytes (Belaoussoff et al., 1998; Wilt, 1965). By connecting to the embryonic circulatory system, and that of the allantois, the yolk blood vessels could now channel nutritious yolk products to the fetus and its primitive erythrocytes could collect oxygen via the chorio-allantoic membrane, bringing these, too, into the embryo via this novel three-way circulatory system.

The primitive streak, which forms mesoderm and endoderm, then systematically distributes these cells throughout the embryo and aligns the mesoderm-derived embryonic vascular system with those of the yolk sac and allantois. In this way, primitive endoderm guarantees the fetus both proper organogenesis and efficient exchange of nutrients, wastes and gases with its environment during gestation and adulthood.

The organizing role of the VE/hypoblast on the circulatory system might not be restricted to vasculogenesis: it has been proposed from work in the chick embryo, from the activity of a mammalian cell line (XEN) and from comparisons with findings in amphibians (Schneider and Mercola, 1999), that an additional function of the hypoblast/AVE might be to induce cardiogenesis in the epiblast (Brown et al., 2010; Matsui et al., 2008; Yatskievych et al., 1997). However, it is not yet clear how this occurs or how it relates to the normal development of the heart in intact embryos.

### Establishing the mouse allantois

Recent experiments in the mouse suggest that PVE is required for the formation of the mouse allantois (Downs et al., 2009). There, in collaboration with what appears to be a posterior extension of the primitive streak, a putative posterior node is established, whose cells direct allantoic elongation to the chorion and contribute to a variety of posterior structures, both embryonic and extra-embryonic (Mikedis and Downs, 2012). Characterization of 'allantois-associated extra-embryonic visceral endoderm' (AX) is in its early stages and thus, its relation to the PVE and other types of VE is not yet clear. Its initial morphology is that of TVE (Bonnevie, 1950; Downs et al., 2009), which gradually transforms into the flattened and molecular profile of EVE. However, the AX might induce the formation of a novel vessel, provisionally referred to as the 'vessel of confluence' (VOC) (Downs, 2009). At the VOC, the three major arterial systems of the amniote amalgamate (Downs et al., 1998; Inman and Downs, 2006) and it has been proposed that the VOC is an amniote innovation (Daane and Downs, 2011), the position of which relative to the embryonic, yolk sac and allantoic circulatory system is aligned with the primitive streak, or axial midline, ensuring the efficient and organized flow of blood to the chorion for exchange with the environment.

### Roles in primordial germ cell (PGC) development

In the chick embryo, PGCs arise in the central epiblast from where they then ingress into the hypoblast before primitive streak formation. The anterior movements of the hypoblast carry these cells to an anterior region, the germinal crescent, underlying the proamnion from where they will eventually colonize the circulation at about the 12-somite stage, and from there migrate to the mesentery and gonads (Ginsburg, 1997; Ginsburg and Eyal-Giladi, 1986; Karagenc et al., 1996; Petitte et al., 1997; Tsunekawa et al., 2000). However, this process appears to be unique to birds. In mouse and other mammals, as well as probably in reptiles, the PGCs are thought

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to arise posteriorly at the embryonic/extra-embryonic junction, where TVE is found (Bonnevie, 1950), and they then colonize the allantoic stalk, from where they migrate into the nearby definitive hindgut endoderm, through the dorsal mesentery and then to the gonads (DeFalco and Capel, 2009). However, it has recently been proposed that the VE influences germ cell specification in mouse by antagonizing Bmp4 signaling in the adjacent ExE (de Sousa Lopes et al., 2004). It is therefore possible that the VE (and the PVE in particular)/hypoblast has more general functions in germ cell development, some of which remain to be uncovered.

## Anamniotes and the hypoblast/AVE

It has been suggested that tissues equivalent to the AVE exist in anamniotes, mainly based on molecular expression patterns (Coolen et al., 2007) and the fact that some functions appear to be the same; for example, 'anterior endoderm' in frog and fish (Pera and De Robertis, 2000; Rodriguez et al., 2001; Schneider and Mercola, 1999; Smithers and Jones, 2002) and the yolk syncytial layer (YSL) in fish (Ho et al., 1999). Two enhancers have been identified for Otx2 that drive its expression in the AVE in mouse and birds (Albazerchi et al., 2007; Kurokawa et al., 2010); these were suggested to have been conserved since the divergence of sarcopterygians and tetrapods but probably co-opted to drive expression in anterior endoderm in amphibians and in AVE in mammals (Kurokawa et al., 2010). It therefore seems likely that genes active in anterior endoderm of anamniotes were co-opted for new functions with the appearance of extra-embryonic tissues in amniotes. In fish embryos, the center of the embryonic shield lies at the edge of the early gastrula-stage embryo. On either side, it extends as a crescent that contains progressively more 'ventral' (i.e. lateral) mesodermal fates. Thus, the amniote primitive streak corresponds to a shield or blastopore that has relocated to the midline, as if pulled from the middle (Arendt and Nübler-Jung, 1999; Voiculescu et al., 2007).

## Conclusions

In summary, we propose that the primitive endoderm (hypoblast/VE) is an amniote innovation that accompanied the slowing down of development, the production of smaller numbers of young and the freedom from having to rely on intracellular sources of nutrition present in the egg cytoplasm. Extra-embryonic tissues nourished the embryo from the outside and allowed them to grow larger at earlier stages. At the same time, the embryo changed from a 'patterning' (subdivision) mode of axis formation, as seen in anamniotes and long germ band insects, to an axial elongation ('blastema') mode more reminiscent of short germ band insects and annelids. The site of gastrulation was repositioned from the periphery (blastopore/embryonic shield of teleosts and frogs) to the midline (primitive streak of amniotes) and this became the growth zone responsible for laying the axis and organizing the relationship between embryonic structures and their placement relative to their extra-embryonic fetal membranes. Placing this structure in the midline can also trap cells destined for the nervous system and skin. Thus, the hypoblast/VE, although present only transiently in the embryo, acquired several functions connected with positioning the primitive streak at the midline, separating neurectodermal and mesendodermal domains, and ensuring vital vascular connections to the environment.

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