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Blastocyst elongation, trophoblastic differentiation, and embryonic pattern formation

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Abstract

The molecular basis of ungulate and non-rodent conceptus elongation and gastrulation remains poorly understood; however, use of state-of-the-art genomic technologies is beginning to elucidate the mechanisms regulating these complicated processes. For instance, transcriptome analysis of elongating porcine concepti indicates that protein synthesis and trafficking, cell growth and proliferation, and cellular morphology are major regulated processes. Furthermore, potential autocrine roles of estrogen and interleukin-1-β in regulating porcine conceptus growth and remodeling and metabolism have become evident. The importance of estrogen in pig is emphasized by the altered expression of essential steroidogenic and trophoblast factors in lagging ovoid concepti. In ruminants, the characteristic mononucleate trophoblast cells differentiate into a second lineage important for implantation, the binucleate trophoblast, and transcriptome profiling of bovine concepti has revealed a gene cluster associated with rapid trophoblast proliferation and differentiation. Gene cluster analysis has also provided evidence of correlated spatiotemporal expression and emphasized the significance of the bovine trophoblast cell lineage and the regulatory mechanism of trophoblast function. As a part of the gastrulation process in the mammalian conceptus, specification of the germ layers and hence definitive body axes occur in advance of primitive streak formation. Processing of the transforming growth factor-β-signaling molecules nodal and BMP4 by specific proteases is emerging as a decisive step in the initial patterning of the pre-gastrulation embryo. The topography of expression of these and other secreted molecules with reference to embryonic and extraembryonic tissues determines their local interaction potential. Their ensuing signaling leads to the specification of axial epiblast and hypoblast compartments through cellular migration and differentiation and, in particular, the specification of the early germ layer tissues in the epiblast via gene expression characteristic of endoderm and mesoderm precursor cells.


Introduction

Proper conceptus development in early gestation is crucial for implantation and maintaining pregnancy to term. Gestational loss can be high during the initial elongation process of the blastocyst, which indicates it is a crucial developmental period; in swine, loss can approach 20% (Anderson 1978, Bennett & Leymaster 1989). Unlike human and mouse blastocysts, the hatched ungulate blastocyst remains detached in the uterus, and transitions through a phase of rapid trophoblast development that dramatically alters the blastocyst morphology prior to implantation. Elongation, i.e., the lengthening and morphological transition of the conceptus’ extraembryonic tissues from a sphere to ovoid to tubule to filament, occurs in all ungulates during peri-implantation and is concomitant with gastrulation (Geisert et al. 1982, Bazer et al. 1993, Hue et al. 2001). The expansion of the trophoblast provides an increased placental surface area to enable maternal:conceptus cross-talk and nutrient exchange that are essential for the survival of the conceptus (Stroband & Van der Lende 1990). Accompanying
elongation is the degradation of the sheath of trophoblasts cells covering the embryonic disc (Rauber’s layer) exposing the cells of the embryonic disc to the maternal milieu (Marrable 1971, Guillomot et al. 2004). In the ewe and cow blastocyst, trophoblast elongation is initiated around gestational day 11 (gd11) and gd12 respectively, and transition from the ovoid to filamentous stage is complete after several days (ewe, gd16; cow, gd18; Bazer et al. 1993, Guillomot et al. 2004). In cattle, trophoblast cells start to elongate at gd14 and the embryonic membrane can extend the entire length of both uterine horns by gd24. The bovine conceptus size increases more than 1000-fold during elongation (Maddock-Hytell et al. 2003) and is accomplished by an increase in cell number and accompanying protein synthesis (Thompson et al. 1998, Degrelle et al. 2005). However, the rate and extent of morphological change of the blastocyst is unsurpassed in pig as the conceptus elongates from an ovoid of <10 mm to a long thin filament >150 mm within ~4 h between gd11 and gd12 (Anderson 1978, Geisert et al. 1982).

Coincident with elongation of the trophoblast in ungulates is the growth and differentiation of the inner cell mass to an embryonic disc (i.e., onset of gastrulation). Microscopic analysis has demonstrated that distinct morphological changes occur in the pig, sheep, and cow embryonic disc (Hue et al. 2001, Guillomot et al. 2004, Blomberg et al. 2006) similar to the ones observed in the rabbit (Viebahn et al. 2002). However, use of the brachyury mesodermal marker, reliable for the detection of embryonic disc polarization and gastrulation state, has demonstrated that there is more asynchrony between the embryonic disc maturation stage and blastocyst morphology in the pig than sheep (Flechon et al. 2004, Guillomot et al. 2004, Blomberg et al. 2006): in contrast to the ovine ovoid conceptus, the mesoderm is already migrating extraembryonically by the ovoid stage and many concepti contain a more mature embryonic disc (Blomberg et al. 2006). However, by the filamentous stage, the developmental disparity is diminished between the species and the primitive streak is apparent in most of the embryonic discs of ungulates (Hue et al. 2001, Guillomot et al. 2004, Blomberg et al. 2006).

As yet, little is known about the initiation signal(s) for elongation or for the molecular interaction between the embryonic disc and extraembryonic tissues that determine successful conceptus development during elongation and, therefore, also enable implantation. Various kinds of molecules most likely participate in the elongation and differentiation processes; however, a clear description of factors and mechanisms involved has been impeded by a limitation of the analytical tools available. In the last decade, genomic technologies have been developed that include specific tools, such as RT-PCR, microarray, serial analysis of gene expression (SAGE), and small interfering RNAs (siRNA) and have helped explore the complex events occurring during the elongation phase (Hashizume et al. 2002, Ponsuksili et al. 2002, Hay et al. 2004, Blomberg et al. 2005). Furthermore, the relative simplicity of the elongating conceptus and the ability to dissect apart the primordial components (embryonic disc and trophoblast) or propagate the different cell types (e.g. epiblast, trophoblast, and hypoblast/primitive endoderm cells) also provide a means to examine genes specific to, or regulated within, a distinct compartment of the embryo. Together, these should aid in the elucidation of physiological processes critical to this stage of development and the contribution of the distinct tissues for proper development.

**Factors and mechanisms influencing the porcine conceptus during elongation**

In pig, the expression of a few genes that encode proteins known to be involved in cellular differentiation, immune modulation, and the maternal recognition of pregnancy have been elucidated over the past few decades either by candidate gene analysis and immunohistochemistry, or more recently, global gene analyses including expressed sequence tag (EST), suppression subtractive hybridization (SSH), microarray, and SAGE (Smith et al. 2001, Ross et al. 2003, Lee et al. 2005, Blomberg et al. 2006). Based on function, some of the most abundant differentially regulated mRNAs during rapid elongation gd11–gd12, interleukin 1-β (IL1B, an inflammatory response mediator), 17-β hydroxysteroid dehydrogenase (regulator of androgen/estrogen synthesis), cytokeratin 8 and 18 (cytoskeletal proteins important for embryonic differentiation), stratifin (a trophoblast protein associated with cell survival, growth, and migration), and ribosomal proteins (protein processing), could have crucial roles in this development period (Smith et al. 2001, Ross et al. 2003, Blomberg et al. 2005). Although less abundant, the presence and differential regulation of specific family members of the retinoid, all trans retinoic acid (ATRA), suggest that this potent embryonic morphogen may also be involved in cellular differentiation and morphogenesis during this period (Gudas 1994, Yelich et al. 1997a).

Failure of ungulate conceptus elongation in vitro indicates that cross-talk between fetal and maternal compartments is critical for the process. Increased synthesis of estrogen (E2), via up-regulation of steroidogenic proteins, and IL1B in the filamentous pig conceptus are thought to provide important signals to the uterus: E2 may be involved in the maternal recognition of pregnancy, while IL1B may be responsible for the suppression of the maternal immune response to prevent conceptus rejection (Geisert et al. 1982, Pusateri et al. 1990, Yelich et al. 1997b, Ross et al. 2003, Blomberg et al. 2005). Additionally, IL1B may also modulate E2 synthesis via IL1B up-regulation of aromatase (Nestler 1993). The concomitant increase in transcription of the E2 receptor-β and IL1B receptor
mRNA in the filamentous conceptus suggests that these factors have an important autocrine role in the development of the conceptus itself, although the exact physiological effect(s) is not fully established (Kowalski et al. 2002, Ross et al. 2003). Furthermore, a recent global proteomic study of the uterine luminal fluid between gd10 and gd13 has indicated that the uterus secretes retinol-binding protein important for the transfer of ATRA to the fetus, and proteins that modulate glycolipids important for cell plasma membrane integrity (Kayser et al. 2006). The effect of glycolipids on plasma membrane extends beyond fluidity to functionality; glycolipids influence the interaction of important adhesion ligands, such as integrins, or growth factors and their respective receptor (Yates & Rampersaud 1998). Focal adhesions mediated by integrins not only enable physical cell:cell anchoring, but these binding sites act as hubs through which important signal transduction may occur to potentially trigger trophoblast differentiation, proliferation, or migration (Burghardt et al. 2002, Das et al. 2002, Jaeger et al. 2005). Both cytokines (including IL1) and growth factors are thought to regulate integrin bioavailability via the up-regulation of its transcription and integrin bioactivity respectively in trophoblasts (Das et al. 2002, Jaeger et al. 2005, Lim et al. 2006). However, to fully appreciate the intricacy of the cross-talk between conceptus and maternal-secreted factors in the pig, the global profile of expressed genes and the identification of the functional network(s) driven by specific factors (such as hormones, cytokines, or growth factors) as well as network overlaps need to be fully established.

Functional analysis by the ingenuity pathway analysis (IPA; Ingenuity Systems, Redwood City, CA, USA) software (http://www.ingenuity.com) provides a tool to characterize biological functions, pathways, or gene networks that are significant (P value via right-tailed Fisher’s exact test) by comparing the number of dataset genes that participate in a given function, pathway, or network to the total number of times those genes appear in all IPA functions, pathways, or networks. The IPA knowledge base contains human, mouse, and rat literature-curated functional data including Gene Ontology terms but the drawback is that genes must be entered with the annotation of those species. To gain a more in-depth understanding of global and differentially regulated physiological processes present in the pig conceptus gd11 through gd12, human orthologs for 5523, 5307, and 5338 porcine transcripts identified previously by SAGE in ovoid, tubular, and filamentous concepti, respectively were analyzed by IPA. A total of 2028 ovoid, 1943 tubular, and 1975 filamentous porcine genes corresponding to human orthologs were identified and 1972, 1881, and 1921 transcripts respectively mapped to IPA biological functions, classical pathways, and networks.

The major classical pathways detected were cell morphology, oxidative phosphorylation, protein synthesis, and cell proliferation. Similarly, the five most significant biological functions at all three stages were protein synthesis, protein trafficking, RNA post-transcriptional modification, molecular transport, and cell growth/proliferation, which overlap with functions identified in ruminants (Hue et al. 2007). An examination of the total number of genes associated with specific molecular functions indicated that cell growth/proliferation was the most prominent (>200 genes/stage), especially at the tubular stage. For years, the initial elongation phase of the porcine conceptus was thought to occur primarily through reorganization and remodeling of the cells of the extraembryonic tissues rather than hyperplasia (Geisert et al. 1982, Pusateri et al. 1990). Recent studies with a cell proliferation marker, and now IPA, indicate that hyperplasia is a very active component of porcine elongation as in other ungulates (Bazer et al. 1993, Blomberg et al. 2006). Analysis of the genes associated with physiological systems development and function identified a large number of genes that regulate cellular assembly/organization (~200 transcripts) or cellular morphology (~150 transcripts; Fig. 1A). For example, the integrin pathway shown in Fig. 1B was well represented by the SAGE transcripts and could be a mechanism for maternal growth factor signaling and cytoskeletal rearrangement/cell motility. Genes associated with cell movement and embryonic, organismal, hematological, and nervous system development were also detected (Fig. 1A). Noteworthy was the marked increase in the number of transcripts involved in the nervous system between the ovoid and more mature tubular or filamentous stages, which coincides with maturation of the embryonic disc. Pathways containing Wnt (Fig. 1B) and TGFβ that have been associated with epiblast polarization, primitive streak formation, and angiogenesis were also identified (Yamaguchi et al. 1999, Ishikawa et al. 2001, Gadue et al. 2006). Analysis of the genes differentially regulated between the different stages indicated that the ovoid:tubular period exhibited the greatest degree of network interaction; eight of the top ten networks interacted with one or more network (Fig. 2A). Networks associated with IL1B and E2 were two of the top five differentially regulated networks in a comparison between concepti at all three stages. Of particular interest was the interface between E2 (network 4), IL1B (network 1), and ATRA (network 5) during the ovoid:tubular transition (Fig. 2A and B). These networks and a fourth are all associated with cellular growth/proliferation, survival, migration, and transformation. This interface between the E2, IL1B, and ATRA networks was not apparent in ovoid:filamentous or tubular:filamentous stage comparisons.
The intersection of the $E_2$-driven network with four of the other networks during the ovoid:tubular transition suggests $E_2$ may have a central role in the transition. In the pig, elongation is asynchronous and lagging embryos are at greater risk of loss (Bazer et al. 1993). The progression of pregnancy through peri-implantation requires controlled estrogen release; increases in estrogen above the normal level at improper times or above physiological levels can terminate gestation or delay conceptus development respectively (Morgan et al. 1987, Geisert et al. 1991, Cardenas et al. 1997). Real-time PCR analysis of lagging (in presence of tubular or filamentous) and normal (all ovoid) 8 mm ovoid concepti revealed that estrogenic and trophoblast-related transcripts were significantly ($P<0.05$) up-regulated in developmentally delayed concepti: the

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**Figure 1** (A) Physiological systems identified in global analysis. A side by side comparison of the total number of genes within ovoid (Ovd), tubular (tub), and filamentous (Fil) from statistically significant ($P<0.05$) physiological functions detected shows that the majority of genes detected are associated with cellular organization. (B) Regulation of embryo mechanisms by integrin. Factors (highlighted in red) involved in cytoskeletal reorganization or ERK/MAPK and growth factor signaling appear to be regulated by integrin in gd11–gd12 elongating concepti embryos, which may suggest the importance of these molecules in evoking cellular restructuring and cell movement during elongation.

relative quantity was $0.732 \pm 0.252$ vs $5.32 \pm 0.532$ (StAR), $1.001 \pm 0.288$ vs $3.576 \pm 0.04$ (aromatase), and $1.125 \pm 0.069$ vs $2.738 \pm 0.172$ (stratifin) for normal versus lagging concepti. Whether the perturbation of estrogenic or trophoblast-specific transcripts is a cause or consequence is not yet established and requires a more thorough evaluation.

The evolvement of bioinformatics resources to characterize the functional relevance of genes is central for the establishment of an in-depth porcine 'systems biology'
platform that can be used to define the physiology in developmental processes like elongation. Clearly, the pig conceptus and endometrium modulate the levels or signal transduction of the three key factors (E2, IL1B, and ATRA) that exhibit a unique interaction during the ovoid to tubular transition. This interaction may be key to porcine elongation and additional studies with the delayed developmental model or dissected primordial tissue may shed more light on their roles.

Global gene expression profiling in bovine trophoblast cell lineage

The morphological changes of the bovine blastocyst during its transition from a sphere to a filament-like tube during gastrulation can probably be matched by the activation of concomitant molecular cascades, but the details of the underlying mechanism(s) remain to be elucidated. Utilization of genomic technologies has enabled the collation of spatiotemporal gene expression profiling during trophoblast cell proliferation and differentiation (cf. Fig. 3). The global gene expression profiling data of the elongating bovine embryo discussed in the subsequent sections indicate two main points. First, trophoblast mononucleate cell (TMC) proliferation and functional differentiation may be induced by specific genes, such as interferon-γ (IFNT), trophoblast Kunitz domain proteins (TKDps), and the transcription factors POU-domain class 5 transcription factor (POUSF1), ERG, and CDX2 that can regulate IFNT and TKDps. Second, the specific expression of transcripts encoding pregnancy-associated glycoproteins (PAGs), prolactin-related proteins (PRPs), and placental lactogen (CSHI) in the trophoblast giant cell (TGC) lineage may not be related to the differentiation of TGCs from TMCs. The activating enhancer-binding protein 2 (AP-2) family and endogenous retroviruses (ERV) may be key factors in trophoblast cell differentiation, however, it remains unclear whether they are involved in the regulation of specific genes within the TGC and/or the differentiation of the TGC. Although there are limited data regarding the differentiation of bovine trophoblast cells, the following sections discuss the putative role of genes based on the regulation of their transcription during elongation/gastrulation.

Blastocyst to tubular period

A comparison of gd7 (blastocyst) and gd14 conceptus transcriptomes by a bovine-specific microarray revealed that ~500 genes, excluding ESTs, were up-regulated between these two periods, whereas only 26 genes were down-regulated (Ushizawa et al. 2004). Most of the up-regulated genes continued to be expressed until around implantation; these included IFNT, TKDp4, and calreticulin. Another interesting gene family was the PAG; at least seven PAGs were expressed. Additional genes exhibiting stage- or tissue-specific expression have also been detected via in situ hybridization and conventional RT-PCR (Degrelle et al. 2005, Ledgard et al. 2006). The production of IFNT and conceptus size are positively correlated; therefore, it may be a key factor in TMC proliferation (Robinson et al. 2006). The POU5F1 transcription factor is considered a pluripotency factor; however, in bovine as well as porcine, POU5F1 is expressed in the differentiated trophoblast cell (Kurosaka et al. 2004, Yadav et al. 2005, Keefer et al. 2007). Other genes like FGFA, NANO1, GATA6, CDX2, EOMES, ETS2, ASCL2, and HAND1 are expressed in the trophoblast lineage (Degrelle et al. 2005, Arnold et al. 2006a), and ASCL2, being a basic helix-loop-helix transcription factor, for example, may be involved in maintaining trophoblast cell proliferation (Arnold et al. 2006a). The spherical blastocyst also expresses proto-oncogenes like FOS, JUN, and HRAS late in their development, and epidermal growth factor (EGF) and transforming growth factor-α (TGFA) induce FOS and MYC transcription (Tetens et al. 2000). Therefore, both EGF and TGFA appear to be essential in early embryonic development in ruminant species. In the mouse and porcine embryo, EGF and TGFA are localized in trophoblast and are considered to have autocrine and paracrine functions that are involved in elongation of the embryo (Dardik et al. 1992, Vaughan et al. 1992, Kliem et al. 1998). Thus, all the factors alluded to above may participate in the initial elongation of the bovine embryo.

Tubular to filamentous period

During gd17–gd19, ~80 up-regulated genes have been detected (Ushizawa et al. 2004). They include tetraspanin (CD9), prosaposin, superoxide dismutase, IFN-induced 35 K protein, and vascular endothelial growth factor (Ushizawa et al. 2004, Pfarrer et al. 2006). During this period, the trophoblast cell lineage mainly comprises TMCs, however, the enhanced expression of PAGs and placental lactogen (CSHI) genes indicate that TGCs, including the trophoblast binucleate cell (BNC), are present during the latter part of this period. Of particular

Figure 3 Gene expression profile during gastrulation in bovine trophoblast cell. Fifty most up-regulated genes on days 14, 17–19, 20–21, and 27–28 compared with gene expression intensity on day 7 were picked up respectively. Finally, 94 genes were arranged in this figure after eliminating overlap genes. Data were analyzed with hierarchical clustering. Three different concepti were used in each day. Embryonic disc was removed before analysis on days 20–28 respectively. Most genes in upper part were expressed from blastocyst up to peri-implantation period. In lower segment, the 22 genes’ expression clearly increased starting just before implantation, so they may be related to TGC cells. 1: day14 conceptus/day7 blastocyst. 2: gd17–19 conceptus/day 7 blastocyst. 3: gd20–21 conceptus/day 7 blastocyst. 4: gd27–28 extraembryonic membrane/day 7 blastocyst. Original data were reported in Ushizawa et al. 2004.
is difficult to determine whether they are tri-nucleate binucleate but does not express CSH1 protein. Although evidence of the expression of CSH1 protein in TMCs does not exist, some cells with ambiguous identity, i.e., in which it is difficult to determine whether they are tri-nucleate or TMCs, express CSH1. The expression of CSH1 has been confirmed in the embryo before its attachment to endometrium, however, the spatial (subcellular) localization of CSH1 protein within the TGC has not been shown (Kessler et al. 1991, Yamada et al. 2002). Furthermore, CD9, a cell-surface protein implicated in cell adhesion, differentiation, and migration, is expressed in the BNC and may participate in the fusion of trophoblast cells and endometrial epithelia (Xiang & MacLaren 2002, Liu et al. 2006). Therefore, the appearance of TGCs during this critical transitional stage and the TGCs’ ability to produce factors, CSH1 and CD9, which enable conceptus:maternal interaction highlight the importance of this differentiated trophoblast cell type.

**Attachment and initial implantation period**

In cattle, implantation starts on gd20, which has been shown in morphological and molecular studies (Wooding 1992, Yamada et al. 2002). Although limited TGC differentiation is detected during the tubular to filamentous transition, it is clear that the most robust differentiation of the TMC to TGC occurs during this period. The trophoblast differentiation mechanism has not been elucidated, however, a recent report indicates that ERVs play a key role in the TMC to TGC differentiation process in the ovine embryo (Dunlap et al. 2006). The expression of ~20 genes is up-regulated at the initiation of implantation and most of them are related to the TGC (Ushizawa et al. 2004). These include CSH1, PAGs, PRPs, BCL2A1, and cathepsin. The TGCs-related genes (i.e., CSH1, PAGs, PRPs, BCL2A1, and cathepsin) may not induce cell differentiation from TMCs to TGCs but rather be involved in the implantation process. This can be surmised because PRP-1 and CSH1 are expressed in trophoblastic regions at sites where implantation is initiated; in particular the PRP-1 transcript and protein are expressed in confined caruncular areas only within the gravid horn (Yamada et al. 2002).

**Placentation period**

After the initiation of implantation, various genes may become involved in the intricate process between fetus and mother. However, only seven additional genes are induced in trophoblast cells during this period (Ushizawa et al. 2004). Various types of genes whose expression during implantation is well known were detected; PAGs, CSH1, PRPs, TKDPs insulin-like growth factor, allograft inflammatory factor-1, and cathepsins (Yamada et al. 2002, Glover & Seidel 2003, MacLean et al. 2003).

**Roles of trophoblast-specific genes from gastrulation to peri-implantation**

Major trophoblast specific gene expression patterns depend on trophoblast cell-type, TMC and TGC (Ushizawa et al. 2004, Degrelle et al. 2005, Hue et al. 2007). The CSH1 factor is a well-known indicator for TGCs; when this gene is expressed, TMCs differentiate to TGCs even if they are not BNC morphologically (Wooding 1992, Nakano et al. 2002). Although PAGs are also considered indicators for TGCs, some specific PAG genes are expressed in TMCs (Xie et al. 1994, Klisch & Leiser 2003). The PRPs, which are members of the growth hormone/prolactin family, are expressed specifically in TGCs and 13 PRPs have been identified. Only PRP-1 may have a role in the adhesion between trophoblast cells and endometrium (Kessler et al. 1991, Yamada et al. 2002, Hashizume et al. 2007).

One of the most highly expressed genes during the gastrulation is TKDP4 and it may be key factor in early trophoblast proliferation (MacLean et al. 2003, Ushizawa et al. 2004). Although IFN is related to TMC proliferation, it is also an important factor for the maternal recognition of pregnancy (Demmers et al. 2001). The mechanisms of trophoblast cell proliferation and differentiation are in coordination with transcription factors, such as cis-elements (Limesand & Anthony 2001, Ushizawa et al. 2007) and epigenetic regulators (Ferguson-Smith et al. 2006). Differentiation into embryonic or trophoblast lineage constitutes alternative routes. There are well-known genes that direct pluripotency in the mouse, such as POU5F1, NANOG, and FGF4. (Simmons & Cross 2005, Hattori et al. 2007, Rossant 2007); however, their roles are unclear in the bovine trophoblast cell lineage. For example, POU5F1 silences trophoblast IFNT transcription in concert with ETS-2 and CDX2 in TMCs, suggesting that these genes are also involved in regulating trophoblast cell proliferation and IFNT expression (Ezashi et al. 2001, Imakawa et al. 2006). On the other hand, the AP-2 family and Sp-1 may participate in the regulation of TGC-specific gene expression, even though the details of this regulation pathway remain to be studied (Limesand & Anthony 2001, Spencer et al. 2004, Ushizawa et al. 2007). In any case, a reduction in the activities of some pluripotency-related genes may be sufficient to permit pluripotent embryonic cells to differentiate into the trophoblast cell lineage. Epigenetic regulation of transcription factors by DNA methylation and histone acetylation have recently been found to regulate imprinted genes, in mice and humans, and some trophoblast related genes may also
Cellular signaling and migration during early axial differentiation in the mammalian blastocyst

In view of the relatively large extraembryonic portion that makes contact with the maternal tissue during elongation, the small principally disc-shaped embryo proper is less frequently considered to play a role in the fetomaternal cross-talk involved in guaranteeing pregnancy success. However, the blueprint of the body plan, i.e., the coordinates of principal body axes, are being established in preparation for the gastrulation process at around this developmental period. Without gastrulation, further development would be a wasteful undertaking in biological terms. Therefore, it is intriguing to think that signals from the embryo at this early point in development may be vital for the survival of the implantation process in order to prevent futile development, such as the possible pathological condition (intrauterine development of extraembryonic tissues alone) called hydatidiform mole, in the human. The present section aims to describe the processes leading to the first patterning in the area of the embryo proper during the time of elongation, attachment, and implantation, the result of which may be important for continuing mammalian development during one of the most critical periods of development.

Cell migration and the emerging body axes in the embryonic disc

While extraembryonic tissues proliferate more or less vigorously (depending on the species under consideration, see above) during the later blastocyst phases, the embryonic disc stays two-layered under the vanishing layer of polar trophoblast, initially (Rauber’s layer: Williams & Biggers 1990). A few hours later, the first dramatic change in morphology is introduced as mesoderm cells are generated and the first germ layer

appears in the longitudinally oriented primitive streak in the posterior half of the embryonic disc. Preceding the appearance of mesoderm and primitive streak, the anterior marginal crescent (AMC) can be seen anteriorly as a more subtle morphological change (Viebahn et al. 1995). Together with an intervening posterior gastrula extension phase, in which the overall morphology of the embryonic disc is changed from a transverse to a longitudinally oriented oval, three phases of initiating gastrulation can be distinguished on the basis of morphological characteristics (Fig. 4B–D, cf. Viebahn 2004). Visual recognition of the relevant disc morphology in the living embryo is particularly helped by the fact that the embryonic disc is integrated into the otherwise uniform trophoblastic surface of the blastocyst. The superficial position of the embryonic disc in the round blastocyst of the rabbit and the AMC per se, as an anterior landmark, has also helped in identifying migratory paths of epiblast cells in relation to the future position of the primitive streak (Viebahn et al. 2002). In the first phase, these cell movements that originate in the posterior half-circular belt of proliferation are directed solely toward the posterior margin and thereby lead to posterior elongation (‘posterior gastrula extension’) of the embryonic disc (Fig. 4E–G and J). During the second phase, and in the newly generated area of the posterior gastrula extension, movements appear to be more complex in that, instead of a simple mass movement toward the posterior pole, individual neighboring cells seem to move in opposite directions (cf. Fig. 4G and H); this contributes to the elongation of the primitive streak in the midline of the embryo (Fig. 4I). These movements are very likely controlled by the non-canonical Wnt signaling cascade through modulation of cell adhesion molecules (E-cadherin) as described for the equivalent developmental period in zebrafish (Ulrich et al. 2005).

While these movements in the epiblast fit movements at later stages (meticulously described by single cell labeling for epiblast movements at post-primitive streak stages in the mouse; Lawson & Pedersen 1992), cell movements in the lower layer (named primitive endoderm in the mouse and hypoblast in non-rodent mammals) are more difficult to observe. The succession of marker gene expression patterns (Hex, Hesxl, Cer, Dkk, gsc and others; Thomas & Beddington 1996, Rivera-Perez & Magnuson 2005, cf. Idkowiak et al. 2004a) suggests that there is a general movement of the hypoblast (primitive endoderm) toward the anterior pole, even beyond the anterior margin. Green fluorescent protein reporter gene constructs coupled to hypoblast-specific marker genes, such as Hex (Rodriguez et al. 2001) or Cerberus like (Cer1; Mesnard et al. 2004), enabled investigations confirming the direction of these movements in the mouse and made experimental analyses possible. These studies demonstrated the dependence of these movements on attracting and repelling effects of TGF-β growth factors, such as nodal and Gdf3, and their
co-receptor cripto or inhibitor lefty also in the mouse (Yamamoto et al. 2004, Chen et al. 2006).

**Molecular blueprint of the body plan**

Sophisticated genetic studies in the mouse have recently disclosed a wealth of different expression patterns in the pre-gastrulation mouse embryo (Tsang et al. 1999, Perea-Gomez et al. 2004, Yamamoto et al. 2004, Rivera-Perez & Magnuson 2005, cf. Ben-Haim et al. 2006, Frankenberg et al. 2007). In general, differential gene expression seems to emerge earlier and in a more complex topographical arrangement in the hypoblast (rabbit; for Dkk1 and Cer1 cf. Fig. 4M and N; mouse: for Hex, Cer1, Lefty cf. Yamamoto et al. 2004) than in the epiblast (rabbit: for Brachyury cf. Fig. 4I; mouse: for Otx2 cf. Perea-Gomez et al. 2001b, for Brachyury cf. Perea-Gomez et al. 2004, Rivera-Perez & Magnuson 2005), which supports the notion that the hypoblast (being replaced by definitive endoderm during gastrulation and, therefore, as an ‘extraembryonic’ tissue of the embryonic disc area) harbors the driving forces for patterning of the overlying (pluripotential) epiblast (Perea-Gomez et al. 2001a, Idkowiak et al. 2004b). However, a description and understanding of these patterns in the wider and general context of mammalian development remains incomplete due to the complex morphology of the rodent egg cylinder caused by the so-called germ layer inversion. Not only does this make visualization of the expression pattern more difficult due to the unavoidable superimposition of opposing parts of the egg cylinder in obligatory side views, but the general validity of the patterns in themselves might also be obscure because the patterns in the proximal epiblast area of the rodent egg cylinder have to be envisaged to be spread out along the periphery of the embryonic disc of the ‘normal’ (i.e., non-rodent) mammal. This is a classical but intriguing case of comparative embryological ‘morphing’, but the relative enlargement of different parts of the proximal epiblast into the continuum of the disc periphery remains, at first sight, undetermined. Luckily, with the advent of in situ gene expression analysis, functional domains rather than morphological characteristics of the typical flat mammalian embryonic disc can now be compared directly with those found in the mouse egg cylinder to solve the problem. Using this functional information, the expression patterns of a few genes, namely Dkk1 and Brachyury, during early gastrulation in rabbit, cow, sheep, or pig as well as mouse lead to the first suggestions as to how the normal mammalian flat embryonic disc was

**Figure 4** (A–D) Initial gastrulation stages as seen in en face views of living rabbit blastocysts between 6.0 and 6.5 d.p.c. under dark-field illumination at low (A) and higher magnification (B–D). The anterior marginal crescent (amc in B) is the initial sign of the axial differentiation typical for gastrulation and defines stage 1. The posterior gastrula extension (pge in C) defines stage 2 and the primitive streak (s in D) defines stage 3. Arrows in B–D point to the posterior border at stage 1 (B) and the remnant of this border at later stages (C and D). Bar: 700 μm in A, 200 μm in B–D. A reprinted from Viebahn C, Mayer B & Hrabe de Angelis M 1995 Signs of the principle body axes prior to primitive streak formation in the rabbit embryo. Anatomy and Embryology 192 159–169 (copyright Elsevier 1995), with kind permission of Springer Science and Business Media. B–D reprinted, with permission, from Viebahn C, Stortz C, Mitchel SA & Blum M 2002 Low proliferative and high migratory activity in the area of the anterior marginal crescent (avm) in the gastrulating rabbit embryo. Development 129 2355–2365. (E–I) Migrating epiblast cells in the posterior half of late pre-streak embryonic discs as demonstrated by labeling intact blastocysts with deposits of DiI suspended in corn oil (nos 1, 2, and 3) and culturing for 12 h. The same embryonic disc before (E) and after (F) suspension culture, under dark-field optics (E and F) and using fluorescence in E and F, 75 μm in G and H, 20 μm in J. E–H and J reprinted, with permission, from Viebahn et al. (2002). (K–Q) Functional compartments at stage 1 in epiblast (K, L, O and P) and hypoblast (M, N and Q) as seen in dorsal views (K, L and M) and 5 μm resin sections following in situ hybridization using specific rabbit cRNA. (K) Expression of the signaling molecule BMP4 is found diffusely distributed within the embryonic disc and in adjacent extraembryonic tissues but it is particularly strong in a narrow band along the anterior circumference. Black dotted line marks the epiblast–trophoblast margin, red dotted line marks the inner border of a peripheral band of epiblast cells, here named the ‘marginal epiblast’. Vertical line indicates position of sagittal section (O) showing strong expression in the epiblast and possibly weak expression in a few hypoblast cells; white bracket h=hypoblast, white bracket e=epiblast. (L) Dark-field view of expression of the mesodermal transcription factor brachyury restricted to the posterior gastrula extension at the posterior pole of the embryonic disc, sparing however, the most peripheral epiblast cells. (M) Sagittal section (position in embryo see vertical line in L) shows brachyury expression confined to the epiblast. Dots and brackets as in L. (M) Cerberus-like expression at the centre and anterior pole of the embryonic disc sparing a peripheral band of cells wider than the one defined by the BMP4 expression domain (in K). Dotted line marks epiblast–trophoblast border of embryonic disc. (N) Clasp-like Dkk1 expression domain at the anterior margin of the disc. Dotted line marks the boundary of a peripheral band as seen in Cerberus-like expression but, in addition, sparing also a central domain of the disc. Vertical line indicates position of sagittal section (Q), which shows Dkk1-expression solely in the hypoblast; green dot marks posterior border of anterior marginal crescent (ave in the mouse), red and black dots and brackets as in L, M, N and Q reprinted from Idkowiak J, Weisheit G, Pitzner J and Viebahn C 2004 Hypoblast controls mesoderm generation and axial patterning in the gastrulating rabbit embryo. Development Genes and Evolution 214 591–605 (copyright 2004 Elsevier), with kind permission of Springer Science and Business Media. (R) Schematic representation (sagittal section in S and U, three-dimensional views in T and V) of compartments in the early mammalian blastocyst deduced from comparing gene expression domains in late blastocysts of rabbit (S and T) and rodent (U and V) embryos; anterior is to the left. U and V are adapted from Beddington et al. 1992. (W) BMP4 expression in trophectoderm of 6.5 d.p.c. mouse egg cylinder (X) Furin expression in trophectoderm of 6.5 d.p.c. mouse egg cylinder. W and X are reprinted from Ben-Haim N, Lu C, Guzman-Ayala M, Pescatore L, Mesnard D, Bischofberger M, Naef F, Robertson EJ & Constam DB 2006 The nodal precursor acting via activin receptors induces mesoderm by maintaining a source of its convertases and BMP4. Developmental Cell 11 313–323 (copyright 2006 Elsevier), with kind permission from Elsevier. Bar in D represents 200 μm in K–N, 20 μm in O–Q, Bar in W represents 20 μm in W and X.

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transformed into the egg cylinder in the case of the rodents. The inverted U shape of the Dkk1 domain is retained (cf. Fig. 3G in Kemp et al. 2005 with Fig. 3G in Idkowiak et al. 2004b, reproduced as Fig. 4N this paper), as is the sickle-like Brachyury expression domain in the epiblast (cf. Fig. 2O–Q in Perea-Gomez et al. 2004 with Fig. 4A–C in Viebahn et al. 2002, s.a. Fig. 4L this paper).

**Extraembryonic signaling for establishing the body plan**

Prior to gastrulation, Brachyury expression seems to unravel differences in the molecular set-up between rodents and other mammals. A ring-like expression pattern in the trophoderm region adjacent to the proximal epiblast (Thomas & Beddington 1996, Perea-Gomez et al. 2004, Rivera-Perez & Magnuson 2005) has no equivalent in other mammalian species (sheep: Guillomot et al. 2004; pig: Flechon et al. 2004; cattle: Hue, personal communication; rabbit: CV unpublished). While this difference points to apparent special requirements of the rodent egg cylinder at the start of gastrulation, comparing the expression pattern of Bmp4 in the early mouse egg cylinder with that of the rabbit, again, to the ring-like region of the early mammalian gastrula, which lies adjacent to the embryo proper. In the mouse, Bmp4 expression is confined to an area commonly referred to as the (extraembryonic) trophoderm (Ben-Haim et al. 2006, cf. Fig. 4W), whereas in the rabbit, Bmp4 appears to be expressed still within the confines of the embryonic–extraembryonic border (Fig. 4K and O). This suggests that the periphery of the rabbit epiblast (generally considered to be extraembryonic and hence part of the embryo proper, Fig. 4S and T) is equivalent to the trophoderm of the mouse (Fig. 4U and V) and could therefore be a tissue with extraembryonic fate and function, i.e. Brachyury expression (Fig. 4L) and extraembryonic mesoderm formation. Apart from this, BMP4 expression in the rabbit reveals an interesting anterior–posterior polarization in this peripheral epiblast (Fig. 4K), so far not suspected to be present in the murine trophoderm. However, the periphery of the embryonic disc also stands out with regard to expression patterns in the hypoblast, where genes involved in axial differentiation (Cer1 and Dkk1) seem to avoid the peripheral extremities of this layer (Idkowiak et al. 2004, Fig. 4M and N this paper). Taken together, these patterns of gene expression in either epiblast or hypoblast of the disc periphery lead us to introduce ‘marginal epiblast’ as a specific term to define this seemingly important domain of the mammalian embryo (Fig. 4S–V).

Intimately connected with BMP4 function is the limited proteolysis and protein-processing function of the subtilisin-like proprotein convertases, Furin and PACE4 on various TGFβ-growth factors, including nodal, recently described by Ben-Haim et al. (2006). Expression of these factors is found, indeed, in trophoblast adjacent to the embryonic disc in cattle (Degrelle et al. 2005) or in the trophoderm part of the egg cylinder (Ben-Haim et al. 2006). However, further studies and functional analysis of these factors will have to be carried out to test the existence of the ‘marginal epiblast’.

**A new trophoblast fate map?**

Intriguing implications of the trophoderm – marginal epiblast equivalence concern adjacent extraembryonic regions of the trophoderm. In the ungulate and rabbit, the placentogenic (and elongating) trophoderm lies laterally adjacent to embryonic disc area (Fig. 4S), while the intervening trophoderm is, at early stages, occupied by Rauber’s layer (Fig. 4R). Rauber’s layer is an entity hitherto not described in the mouse but equivalent cells may possibly be present in rodents rather early as a short-lived set of trophoblast cells in the centre of the polar trophoderm (grey cells in Fig. 4R); in rodents, these cells may be eradicated earlier than in ungulates and rabbits and may be replaced by the trophoderm ‘precociously’ expanding to guarantee early rodent implantation. As a very short-lived cell population, Rauber’s layer may, therefore, have remained undetected so far in rodents.

The concept of short-lived Rauber’s cells in rodents may be a simple model to explain the gross differences in the topography of implantation amongst mammals: most mammalian species up to lower primates behave as the ungulates and appear to generate placentogenic trophoblast at the lateral or abembryonic part of the blastocyst, while others, such as rodents and higher primates including man, initiate placentation at the embryonic pole of the blastocyst. This discrepant mode of placenta-tion was classically explained by dichotomous behavior of ‘mural’ and ‘polar’ trophoblast at the blastocyst stage (cf. Mossman 1971). Introducing the concept of apoptotic trophoblast (Rauber’s) cells for all mammals, however, (1) assumes a uniform fate map of all subpopulations of the early trophoderm (cf. Fig. 4R with S and U respectively) and (2) may explain the grossly different modes of placenta-tion by simple differences in the timing of proliferation and apoptosis in the trophoblast adjacent to the inner cell mass: concerning the first issue, the uniform fate map, a belt-like area of trophoblast cells bridging the border of classical polar and mural trophoblast may contribute to placentogenic trophoblast (forming the ectoplacental cone in rodents and the chorion frondosum, the main villous placenta, in man, for example), while in most other mammals the remaining polar trophoblast constitutes the founder population of Rauber’s layer and the remaining mural trophoblast turns into the trophoblast of the chorion laeve (non-placentogenic trophoblast). With regard to the regulation of the timing, the second issue, proliferation of placentogenic trophoblast and apoptosis of Rauber’s cells may be switched on late permitting (a) expansion of the inner cell mass to a disc shape and leading (b) to a separation of the placentogenic trophoblast into a ring shape.
surrounding the embryonic disc in late implanting species such as the rabbit (cf. arrow between panels R and S in Fig. 4). In contrast, early proliferation and apoptosis of the respective cells in early implanting species ‘draws’ the placentogenic trophoblast cells together to cover the inner cell mass and to form a (common) ectoplacental cone (in rodents, cf. arrow between panels R and U in Fig. 4) or the (undivided) ‘polar trophoblast’ placenta (in higher primates and man).

Experimental investigations, including SSH and differential gene expression profiling using the subcomponents of the trophoblast either described in this hypothetical fate map or as outlined at the beginning of this section (marginal epiblast/trophectoderm versus trophoblast/ectoplacental cone), may elucidate molecular differences in trophoblast development responsible for both embryonic development (gastrulation) and sustaining pregnancy. The issue of differential timing in trophoblast proliferation and apoptosis may, indeed, hold the key for describing success and failure, and the evolution, of implantation.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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