Focus on Mammalian Embryogenomics

Blastocyst elongation, trophoblastic differentiation, and embryonic pattern formationLeAnn Blomberg, Kazuyoshi Hashizume¹ and Christoph Viebahn²

Animal Biosciences and Biotechnology Laboratory, USDA Agricultural Research Service, Beltsville, Maryland 20705, USA, ¹Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, Morioka, Iwate 020-8550, Japan and ²Department of Anatomy and Embryology, Göttingen University, Kreuzberggring 36, 37075 Göttingen, Germany

Correspondence should be addressed to C Viebahn; Email: cviebah@gwdg.de

L Blomberg, K Hashizume and C Viebahn contributed equally to this work

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.

Reproduction (2008) 135 181–195

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

This article was presented at the 2nd International Meeting on Mammalian Embryogenomics, 17–20 October 2007. The Co-operative Research Programme: Biological Resource Management for Sustainable Agricultural Systems of The Organisation for Economic Co-operation and Development (OECD) has supported the publication of this article. The meeting was also sponsored by Le conseil Régional Ile-de-France, the Institut National de la Recherche Agronomique (INRA), Cogenics-Genome Express, Eurogentec, Proteigene, Sigma-Aldrich France and Diagenode sa

elongation is the degradation of the sheath of trophoblasts cells covering the embryonic disc (Raubers' 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 (Maddox-Hyttel *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 (E_2), via up-regulation of steroidogenic proteins, and IL1B in the filamentous pig conceptus are thought to provide important signals to the uterus: E_2 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 E_2 synthesis via IL1B up-regulation of aromatase (Nestler 1993). The concomitant increase in transcription of the E_2 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 TGFB 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.

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.

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

Figure 2 Networks regulated during the ovoid to tubular transition. The IPA network analysis provides the functional relationship/interaction of protein:protein, protein:nucleic acid, or protein:chemical (e.g. E₂) within a dataset. (A) The interface between eight of the significant ($P < 0.05$) ovd:tub networks is shown by the solid line. Networks 4 and 5 are driven primarily by E₂ and ATRA respectively, whereas Network 1 is centered around the upstream regulation of IL1B and downstream regulation by IL1B. (B) Modulation of Cellular Development, Proliferation and Cellular Energy by β -estradiol. Expansion of the E₂ driven network denotes the factors (IPA node types) regulated indirectly by E₂ and how they interface (IPA edge types). Color codes show factors with increased expression at ovoid (red) and tubular (green) stages; the more intense the color the more highly expressed the factor is relative to all the factors identified in the specific SAGE library. The boxed factors depict the intersection of the E₂ network with ATRA (red) and IL1B (blue) networks.

relative quantity was 0.732 ± 0.252 vs 5.32 ± 0.532 (StAR), 1.001 ± 0.288 vs 3.576 ± 0.04 (aromatase), and 1.125 ± 0.069 vs 2.738 ± 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 (E_2 , 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- τ (*IFNT*), trophoblast Kunitz domain proteins (TKDPs), and the transcription factors POU-domain class 5 transcription factor (POU5F1), 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 (*CSH1*) 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 (ERVs) 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 also expressed in the differentiated trophoblast cell (Kurosaka *et al.* 2004, Yadav *et al.* 2005, Keefer *et al.* 2007). Other genes like *FGF4*, *NANOG*, *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 (TGF α) induce *FOS* and *MYC* transcription (Tetens *et al.* 2000). Therefore, both EGF and TGF α appear to be essential in early embryonic development in ruminant species. In the mouse and porcine embryo, EGF and TGF α are localized in trophoblast and are considered to have both 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 (*CSH1*) 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: day17–19 conceptus/day 7 blastocyst. 3: day20–21 conceptus/day 7 blastocyst. 4: day27–28 extraembryonic membrane/day 7 blastocyst. Original data were reported in Ushizawa *et al.* 2004.

interest is CSH1, which is thought to regulate morphogenic and differentiation events within the maternal endometrium to promote conceptus:maternal interaction (Wooding 1992). The TGC population comprises at least two different cell types: (1) the BNC which is binucleate and expresses CSH1 protein, and (2) the cell that is 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 *IFNT* 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 mouse and humans, and some trophoblast related genes may also

be involved in epigenetic regulation: reports suggest *NANOG*, *POU5F1*, sal-like protein 3 (*SALL3*), and serpin inhibitor clade B member 5 (*SERPINB5*) are involved in epigenetic regulation that influence trophoblast cell proliferation, differentiation, and invasion (Ohgane *et al.* 2004, Dokras *et al.* 2006, Ferguson-Smith *et al.* 2006, Tomikawa *et al.* 2006). However, in the bovine embryo, there is again only limited evidence regarding epigenetic modification of trophoblast-specific genes (Kremensky *et al.* 2006a, 2006b). Cattle generated by somatic cell nuclear transfer demonstrate various abnormalities, including placental malformation, which are thought to be caused by aberrant epigenetic regulation (Hashizume *et al.* 2002, Hall *et al.* 2005, Arnold *et al.* 2006b, Rybouchkin *et al.* 2006), and suggests that trophoblast cell differentiation is under the control of epigenetic regulation as well.

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 (Raubers' 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*, *Hesx1*, *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. 4L; 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, Mitchell SA & Blum M 2002 Low proliferative and high migratory activity in the area of *Brachyury* expressing mesoderm progenitor cells 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 Dil 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 microscopy (G, H and J). Hatched line in G indicates orientation and length of frozen section segment shown in J. (I) composite drawing indicating the convergence and extension movements (blue arrows) at stage 3 resulting from cell movements in the epiblast (red arrows) at stage 2. Bar: 300 µm 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. (P) 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 domain sparing 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 of border of anterior marginal crescent (AVE in the mouse), red and black dots and brackets as in L. M, N and Q are reprinted from Idkowiak J, Weisheit G, Plitzner 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.

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 Ildkowiak *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 trophoblast region adjacent to the proximal epiblast of the rodent (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 points, 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) trophoblast (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 intraembryonic and hence part of the embryo proper, Fig. 4S and T) is equivalent to the trophoblast 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 trophoblast. 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 (Ildkowiak *et al.* 2004, cf. 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 TGF β -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

trophoblast 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 trophoblast – marginal epiblast equivalence concern adjacent extraembryonic regions of the trophoblast. In the ungulate and rabbit, the placentogenic (and elongating) trophoblast lies laterally adjacent to embryonic disc area (Fig. 4S), while the intervening trophoblast 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 trophoblast (grey cells in Fig. 4R); in rodents, these cells may be eradicated earlier than in ungulates and rabbits and may be replaced by the trophoblast ‘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 placentation 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 trophoblast (cf. Fig. 4R with S and U respectively) and (2) may explain the grossly different modes of placentation 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 subcompartments 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.

Funding

This study was supported in part by Hoga-kenkyu (19658101); Kiban B (17380172) of the Ministry of Education, Science and Technology; the Ministry of Agriculture, Forestry and Fisheries, Japan, the National Institute of Agrobiological Sciences and Deutsche Forschungsgemeinschaft (Vi151/4-Vi151/8). Ingenuity Pathway Analysis was supported by USDA ARS CRIS Project No. 1265-31000-082. This article is based on research presented at the 2nd International Meeting on Mammalian Embryogenomics, which was sponsored by the Organisation for Economic Co-operation and Development (OECD), Le conseil Régional Ile-de-France, the Institut National de la Recherche Agronomique (INRA), Cogenics-Genome Express, Eurogentec, Proteigene, Sigma-Aldrich France and Diagenode sa. All authors received funding from the OECD to attend the meeting. All authors have collaborative research projects with INRA, which do not involve funding.

Acknowledgements

The authors express their sincere thanks to Dr Keiichiro Kizaki of Iwate University; Drs Toru Takahashi, Kouichi Ushizawa and Misa Hosoe of the National Institute of Agrobiological Sciences for their assistance in the cDNA microarray assembly and data analysis. The authors thank Lori Schreier for assistance with animal management and genomic analysis, Drs Robert Li and George Liu for help with bioinformatic analysis of SAGE tag data, and Dr John McMurtry for review of the manuscript. All functional pathways and networks were generated through the use of Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com).

References

- Anderson LL 1978 Growth, protein content and distribution of early pig embryos. *Anatomical Record* **190** 143–153.
- Arnold DR, Lefebvre R & Smith LC 2006a Characterization of the placenta specific bovine mammalian achate scute-like homologue 2 (Mash2) gene. *Placenta* **27** 1124–1131.
- Arnold DR, Bordinon V, Lefebvre R, Murphy BD & Smith LC 2006b Somatic cell nuclear transfer alters peri-implantation trophoblast differentiation in bovine embryos. *Reproduction* **132** 279–290.
- Bazer FW, Geisert RD & Zavy MT 1993 Fertilization, cleavage, and implantation. In *Reproduction in Farm Animals*, 6 edn, pp 88–212. Ed. ESE Edby Hafez. Philadelphia, Pennsylvania: Lea and Febiger.
- Beddington RSP, Jaenisch R, Smith JC, McLaren AL, Lawson KA & Herrmann BG 1992 Three-dimensional representation of gastrulation in the mouse. In *CIBA Foundation Symposium 165 – Postimplantation Development in the Mouse*, pp 55–60. Eds DJ Chadwick & J Marsh. Chichester: Wiley.
- 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.
- Bennett GL & Leymaster KA 1989 Integration of ovulation rate, potential embryonic viability and uterine capacity into a model of litter size in swine. *Journal of Animal Science* **6** 1230–1241.
- Blomberg LA, Long EL, Sonstegard TS, Van Tassell CP, Dobrinsky JR & Zuelke KA 2005 Serial analysis of gene expression during elongation of the peri-implantation porcine trophectoderm (conceptus). *Physiological Genomics* **20** 188–194.
- Blomberg LA, Garrett WM, Guillomot M, Miles JR, Sonstegard TS, Van Tassell CP & Zuelke KA 2006 Transcriptome profiling of the tubular porcine conceptus identifies the differential regulation of growth and developmentally associated genes. *Molecular Reproduction and Development* **73** 1491–1502.
- Burghardt RC, Johnson GA, Jaeger LA, Ka H, Garlow JE, Spencer TE & Bazer FW 2002 Integrins and extracellular matrix proteins at the maternal–fetal interface in domestic animals. *Cells, Tissues, Organs* **172** 202–217.
- Cardenas H, Trout WE, Simmen RC & Pope WF 1997 Short-term effects of exogenous estradiol-17 beta on blastocyst development during the period of elongation in swine. *Animal Reproduction Science* **47** 123–135.
- Chen C, Ware SM, Sato A, Houston-Hawkins DE, Habas R, Matzuk MM, Shen MM & Brown CW 2006 The Vg1-related protein Gdf3 acts in a Nodal signaling pathway in the pre-gastrulation mouse embryo. *Development* **133** 319–329.
- Dardik A, Smith RM & Schultz RM 1992 Colocalization of transforming growth factor- α and a functional epidermal growth factor receptor (EGFR) to the inner cell mass and preferential localization of the EGFR on the basolateral surface of the trophectoderm in the mouse blastocyst. *Developmental Biology* **154** 396–409.
- Das C, Kumar VS, Gupta S & Kumar S 2002 Network of cytokines, integrins and hormones in human trophoblast cells. *Journal of Reproductive Immunology* **53** 257–268.
- Degrelle SA, Campion E, Cabau C, Piumi F, Reinaud P, Richard C, Renard JP & Hue I 2005 Molecular evidence for a critical period in mural trophoblast development in bovine blastocysts. *Developmental Biology* **288** 448–460.
- Demmers KJ, Derecka K & Flint A 2001 Trophoblast interferon and pregnancy. *Reproduction* **121** 41–49.
- Dokras A, Coffin J, Field L, Frakes A, Lee H, Madan A, Nelson T, Ryu GY, Yoon JG & Madan A 2006 Epigenetic regulation of maspin expression in the human placenta. *Molecular Human Reproduction* **12** 611–617.
- Dunlap KA, Palmarini M, Varela M, Burghardt RC, Hayashi K, Farmer JL & Spencer TE 2006 Endogenous retroviruses regulate periimplantation placental growth and differentiation. *PNAS* **103** 14390–14395.
- Ezashi T, Ghosh D & Roberts RM 2001 Repression of Ets-2-induced transactivation of the tau interferon promoter by Oct-4. *Molecular and Cellular Biology* **21** 7883–7891.
- Ferguson-Smith AC, Moore T, Detmar J, Lewis A, Hemberger M, Jammes H, Kelsey G, Roberts CT, Jones H & Constancia M 2006 Epigenetics and imprinting of the trophoblast – a workshop report. *Placenta* **27** (Supplement A) S122–S126.

- Flechon JE, Degrouard J & Flechon B 2004 Gastrulation events in the prestreak pig embryo: ultrastructure and cell markers. *Genesis* **38** 13–25.
- Frankenberg S, Smith L, Greenfield A & Zernicka-Goetz M 2007 Novel gene expression patterns along the proximo-distal axis of the mouse embryo before gastrulation. *BMC Developmental Biology* **15** 8.
- Gadue P, Huber TL, Paddison PJ & Keller GM 2006 Wnt and TGF- β signaling are required for the induction of an *in vitro* model of primitive streak formation using embryonic stem cells. *PNAS* **102** 16806–16811.
- Geisert RD, Brookbank JW, Roberts RM & Bazer FW 1982 Establishment of pregnancy in the pig: II. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. *Biology of Reproduction* **27** 941–955.
- Geisert RD, Morgan GL, Zavy MT, Blair RM, Gries LK, Cox A & Yellin T 1991 Effect of asynchronous transfer and oestrogen administration on survival and development of porcine embryos. *Journal of Reproduction and Fertility* **93** 475–481.
- Glover MD & Seidel GE 2003 Increased messenger RNA for allograft inflammatory factor-1, LERK-5, and a novel gene in 17.5-day relative to 15.5-day bovine embryos. *Biology of Reproduction* **69** 1002–1012.
- Gudas LJ 1994 Retinoids and vertebrate development. *Journal of Biological Chemistry* **269** 15399–15402.
- Guillomot M, Turbe A, Hue I & Renard JP 2004 Staging of ovine embryos and expression of the T-box genes Brachyury and Eomesodermin around gastrulation. *Reproduction* **127** 491–501.
- Hall VJ, Ruddock NT & French A 2005 Expression profiling of genes crucial for placental and preimplantation development in bovine *in vivo*, *in vitro*, and nuclear transfer blastocysts. *Molecular Reproduction and Development* **72** 16–24.
- Hashizume K, Ishiwata H, Kizaki K, Yamada O, Takahashi T, Imai K, Patel OV, Akagi S, Shimizu M, Takahashi S *et al.* 2002 Implantation and placental development in somatic cell clone recipient cows. *Cloning and Stem Cells* **4** 197–209.
- Hashizume K, Ushizawa K, Patel OV, Kizaki K, Imai K, Yamada O, Nakano H & Takahashi T 2007 Gene expression and maintenance of pregnancy in bovine: roles of trophoblastic binucleate cell-specific molecules. *Reproduction, Fertility, and Development* **19** 79–90.
- Hattori N, Imao Y, Nishino K, Hattori N, Ohgane J, Yagi S, Tanaka S & Shiota K 2007 Epigenetic regulation of Nanog gene in embryonic stem and trophoblast stem cells. *Genes to Cells* **12** 387–396.
- Hay DC, Sutherland L, Clark J & Burdon T 2004 Oct-4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells. *Stem Cells* **22** 225–235.
- Hue I, Renard JP & Viebahn C 2001 Brachyury is expressed in gastrulating bovine embryos well ahead of implantation. *Development Genes and Evolution* **211** 157–159.
- Hue I, Degrelle SA, Campion E & Renard JP 2007 Gene expression in elongating and gastrulating embryos from ruminants. *Society of Reproduction and Fertility* **64** 365–377.
- Idkowiak J, Weisheit G & Viebahn C 2004a Polarity in the rabbit embryo. *Seminars in Cell and Developmental Biology* **15** 607–617.
- Idkowiak J, Weisheit G & Viebahn C 2004b Hypoblast controls mesoderm generation and axial patterning in the gastrulating rabbit embryo. *Development Genes and Evolution* **214** 591–605.
- Imakawa K, Kim MS, Matsuda-Minehata F, Ishida S, Iizuka M, Suzuki M, Chang KT, Echternkamp SE & Christenson RK 2006 Regulation of the ovine interferon-tau gene by a blastocyst-specific transcription factor, Cdx2. *Molecular Reproduction and Development* **73** 559–567.
- Ishikawa T, Tamai Y, Zorn AM, Yoshida H, Seldin MF, Nishikawa S & Taketo MM 2001 Mouse Wnt receptor gene Fzd5 is essential for yolk sac and placental angiogenesis. *Development* **128** 25–33.
- Jaeger LA, Spiegel AK, Ing NH, Johnson GA, Bazer FW & Burghardt RC 2005 Functional effects of transforming growth factor beta on adhesive properties of porcine trophoblast. *Endocrinology* **146** 3933–3942.
- Kayser JPR, Kim JG, Cerny RL & Vallet JL 2006 Global characterization of porcine intrauterine proteins during early pregnancy. *Reproduction* **131** 379–388.
- Keefer CL, Pant D, Blomberg L & Talbot NC 2007 Challenges and prospects for the establishment of embryonic stem cell lines of domesticated ungulates. *Animal Reproduction Science* **98** 147–168.
- Kemp C, Willems E, Abdo S, Lambiv L & Leyns L 2005 Expression of all Wnt genes and their secreted antagonists during mouse blastocyst and postimplantation development. *Developmental Dynamics* **233** 1064–1075.
- Kessler MA, Duell TM & Schuler LA 1991 Expression of prolactin-related hormones in the early bovine conceptus, and potential for paracrine effect on the endometrium. *Endocrinology* **129** 1885–1895.
- Kliem A, Tetens F, Klonisch T, Grealy M & Fischer B 1998 Epidermal growth factor receptor and ligands in elongating bovine blastocysts. *Molecular Reproduction and Development* **51** 402–412.
- Klisch K & Leiser R 2003 In bovine binucleate trophoblast giant cells, pregnancy-associated glycoproteins and placental prolactin-related protein-I are conjugated to asparagine-linked N-acetylgalactosaminyl glycans. *Histochemistry and Cell Biology* **119** 211–217.
- Kowalski AA, Graddy LG, Vale-Cruz DS, Choi I, Katzenellenbogen BS, Simmen FA & Simmen RC 2002 Molecular cloning of porcine estrogen receptor-beta complementary DNAs and developmental expression in periimplantation embryos. *Biology of Reproduction* **66** 760–769.
- Kremensky M, Kremenska Y, Suzuki M, Imai K, Takahashi S, Hashizume K, Yagi S & Shiota K 2006a DNA methylation profiles of donor nuclei cells and tissues of cloned bovine fetuses. *Journal of Reproduction and Development* **52** 259–266.
- Kremensky M, Kremenska Y, Suzuki M, Imai K, Takahashi S, Hashizume K, Yagi S & Shiota K 2006b Epigenetic characterization of the CpG islands of bovine Leptin and POU5F1 genes in cloned bovine fetuses. *Journal of Reproduction and Development* **52** 277–285.
- Kurosaka S, Eckardt S & McLaughlin KJ 2004 Pluripotent lineage definition in bovine embryos by Oct4 transcript localization. *Biology of Reproduction* **71** 1578–1582.
- Lawson KA & Pedersen RA 1992 Clonal analysis of cell fate during gastrulation and early neurulation in the mouse. In *CIBA Foundation Symposium* 165, pp 3–26. Eds DJ Chadwick & J Marsh. Chichester: Wiley.
- Ledgard AM, Lee RS & Peterson AJ 2006 Expression of genes associated with allantois emergence in ovine and bovine conceptuses. *Molecular Reproduction and Development* **73** 1084–1093.
- Lee SH, Zhao SH, Recknor JC, Nettleton D, Orley S, Kang SK, Lee BC, Hwang WS & Tuggle CK 2005 Transcriptional profiling using a novel cDNA array identifies differential gene expression during porcine embryo elongation. *Molecular Reproduction and Development* **71** 129–130.
- Lim JJ, Lee DR, Song HS, Kim KS, Yoon TK, Gye MC & Kim MK 2006 Heparin-binding epidermal growth factor (HB-EGF) may improve embryonic development and implantation by increasing vitronectin receptor (integrin α 5 β 3) expression in peri-implantation mouse embryos. *Journal of Assisted Reproduction and Genetics* **23** 111–119.
- Limesand SW & Anthony RV 2001 Novel activator protein-2 α splice-variants function as transactivators of the ovine placental lactogen gene. *European Journal of Biochemistry* **268** 2390–2401.
- Liu WM, Cao YJ, Yang YJ, Li J, Hu Z & Duan EK 2006 Tetraspanin CD9 regulates invasion during mouse embryo implantation. *Journal of Molecular Endocrinology* **36** 121–130.
- MacLean JA II, Chakrabarty A, Xie S, Bixby JA, Roberts RM & Green JA 2003 Family of Kunitz proteins from trophoblast: expression of the trophoblast Kunitz domain proteins (TKDP) in cattle and sheep. *Molecular Reproduction and Development* **65** 30–40.
- Maddox-Hyttel P, Alexopoulos NI, Vajta G, Lewis I, Rogers P, Cann L, Callesen H, Tveden-Nyborg P & Trounson A 2003 Immunohistochemical and ultrastructural characterization of the initial post-hatching development of bovine embryos. *Reproduction* **125** 607–623.
- Marrable AW 1971 *The Embryonic Pig: a Chronological Account*, pp 14–23. New York: Pitman Publishing.
- Mesnard D, Filipe M, Belo JA & Zernicka-Goetz M 2004 The anterior-posterior axis emerges respecting the morphology of the mouse embryo that changes and aligns with the uterus before gastrulation. *Current Biology* **14** 184–196.
- Morgan GL, Geisert RD, Zavy MT & Fazleabas AT 1987 Development and survival of pig blastocysts after oestrogen administration on day 9 or days 9 and 10 of pregnancy. *Journal of Reproduction and Fertility* **80** 133–141.
- Mossman HW 1971 Orientation and site of attachment of the blastocyst: a comparative study. In *The Biology of the Blastocyst*, pp 49–57. Ed. RJ Blandau. Chicago: Univ Chicago Press.

- Nakano H, Shimada A, Imai K, Takahashi T & Hashizume K 2002 Association of Dolichos biflorus lectin binding with full differentiation of bovine trophoblast cells. *Reproduction* **124** 581–592.
- Nestler JE 1993 Interleukin-1 stimulates the aromatase activity of human placental cytotrophoblasts. *Endocrinology* **132** 566–570.
- Ohgane J, Wakayama T, Senda S, Yamazaki Y, Inoue K, Ogura A, Marh J, Tanaka S, Yanagimachi R & Shiota K 2004 The Sall3 locus is an epigenetic hotspot of aberrant DNA methylation associated with placentomegaly of cloned mice. *Genes to Cells* **9** 253–260.
- Perea-Gomez A, Rhinn M & Ang SL 2001a Role of the anterior visceral endoderm in restricting posterior signals in the mouse embryo. *International Journal of Developmental Biology* **45** 311–320.
- Perea-Gomez A, Lawson KA, Rhinn M, Zakin L, Brulet P, Mazan S & Ang SL 2001b Otx2 is required for visceral endoderm movement and for the restriction of posterior signals in the epiblast of the mouse embryo. *Development* **128** 753–765.
- Perea-Gomez A, Camus A, Moreau A, Grieve K, Moneron G, Dubois A, Cibert C & Collignon J 2004 Initiation of gastrulation in the mouse embryo is preceded by an apparent shift in the orientation of the anterior-posterior axis. *Current Biology* **14** 197–207.
- Pfarrer CD, Ruziwa SD, Winther H, Callesen H, Leiser R, Schams D & Dantzer V 2006 Localization of vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 and VEGFR-2 in bovine placentomes from implantation until term. *Placenta* **27** 889–898.
- Ponsuksili S, Tesfaye D, El-Halawany N, Schellander K & Wimmers K 2002 Stage-specific expressed sequence tags obtained during preimplantation bovine development by differential display RT-PCR and suppression subtractive hybridization. *Prenatal Diagnosis* **22** 1135–1142.
- Pusateri AE, Rothschild MF, Warner CM & Ford SP 1990 Changes in morphology, cell number, cell size and cellular estrogen content of individual littermate pig conceptuses on days 9 to 13 of gestation. *Journal of Animal Science* **68** 3727–3735.
- Rivera-Perez JA & Magnuson T 2005 Primitive streak formation in mice is preceded by localized activation of Brachyury and Wnt3. *Developmental Biology* **288** 363–371.
- Robinson RS, Fray MD, Wathes DC, Lamming GE & Mann GE 2006 *In vivo* expression of interferon tau mRNA by the embryonic trophoblast and uterine concentrations of interferon tau protein during early pregnancy in the cow. *Molecular Reproduction and Development* **73** 470–474.
- Rodriguez TA, Casey ES, Harland RM, Smith JC & Beddington RS 2001 Distinct enhancer elements control Hex expression during gastrulation and early organogenesis. *Developmental Biology* **234** 304–316.
- Ross JW, Ashworth MD, Hurst AG, Malayer JR & Geisert RD 2003 Analysis and characterization of differential gene expression during rapid trophoblastic elongation in the pig using suppression subtractive hybridization. *Reproductive Biology and Endocrinology* **1** 23–34.
- Rossant J 2007 Stem cells and lineage development in the mammalian blastocyst. *Reproduction, Fertility, and Development* **19** 111–118.
- Rybouchkin A, Kato Y & Tsunoda Y 2006 Role of histone acetylation in reprogramming of somatic nuclei following nuclear transfer. *Biology of Reproduction* **74** 1083–1089.
- Simmons DG & Cross JC 2005 Determinants of trophoblast lineage and cell subtype specification in the mouse placenta. *Developmental Biology* **284** 12–24.
- Smith TP, Fahrenkrug SC, Rohrer GA, Simmen FA, Rexroad CE & Keele JW 2001 Mapping of expressed sequence tags from a porcine early embryonic cDNA library. *Animal Genetics* **32** 66–72.
- Spencer TE, Johnson GA, Bazer FW & Burghardt RC 2004 Implantation mechanisms: insights from the sheep. *Reproduction* **128** 657–668.
- Stroband HW & Van der Lende T 1990 Embryonic and uterine development during pregnancy. *Journal of Reproduction and Fertility* **40** 261–277.
- Tetens F, Kliem A, Tschedschilsuren G, Navarrete Santos A & Fischer B 2000 Expression of proto-oncogenes in bovine preimplantation blastocysts. *Anatomy and Embryology* **201** 349–355.
- Thomas PQ & Beddington RSP 1996 Anterior primitive endoderm may be responsible for patterning the anterior neural plate in the mouse embryo. *Current Biology* **6** 1487–1496.
- Thompson JG, Sherman AN, Allen NW, McGowan LT & Tervit HR 1998 Total protein content and protein synthesis within pre-elongation stage bovine embryos. *Molecular Reproduction and Development* **50** 139–145.
- Tomikawa J, Fukatsu K, Tanaka S & Shiota K 2006 DNA methylation-dependent epigenetic regulation of dimethylarginine dimethylaminohydrolase 2 gene in trophoblast cell lineage. *Journal of Biological Chemistry* **281** 12163–12169.
- Tsang TE, Kinder SJ & Tam PP 1999 Experimental analysis of the emergence of left-right asymmetry of the body axis in early postimplantation mouse embryos. *Cellular and Molecular Biology* **45** 493–503.
- Ulrich F, Krieg M, Schotz EM, Link V, Castanon I, Schnabel V, Taubenberger A, Mueller D, Puech PH & Heisenberg CP 2005 Wnt11 functions in gastrulation by controlling cell cohesion through Rab5c and E-cadherin. *Developmental Cell* **9** 555–564.
- Ushizawa K, Herath CB, Kaneyama K, Shiojima S, Hirasawa A, Takahashi T, Imai K, Ochiai K, Tokunaga T, Tsunoda Y *et al.* 2004 cDNA microarray analysis of bovine embryo gene expression profiles during the pre-implantation period. *Reproductive Biology and Endocrinology* **2** 77.
- Ushizawa K, Takahashi T, Hosoe M, Ishiwata H, Kaneyama K, Kizaki K & Hashizume K 2007 Global gene expression analysis and regulation of the principal genes expressed in bovine placenta in relation to the transcription factor AP-2 family. *Reproductive Biology and Endocrinology* **5** 17.
- Vaughan TJ, James PS, Pascall JC & Brown KD 1992 Expression of the genes for TGF alpha, EGF and the EGF receptor during early pig development. *Development* **116** 663–669.
- Viebahn C 2004 Gastrulation in the rabbit. In *Gastrulation*, pp 263–274. Ed. CDS Stern. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Viebahn C, Mayer B & Hrabé de Angelis M 1995 Signs of the principal body axes prior to primitive streak formation in the rabbit embryo. *Anatomica Embryologica* **192** 159–169.
- Viebahn C, Stortz C, Mitchell SM & Blum M 2002 Low proliferative and high migratory activity in the area of Brachyury expressing mesoderm progenitor cells in the gastrulating rabbit embryo. *Development* **129** 2355–2365.
- Williams BS & Biggers JD 1990 Polar trophoblast (Raubers' layer) of the rabbit blastocyst. *Anatomical Record* **227** 211–222.
- Wooding FB 1992 Current topic: the synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. *Placenta* **13** 101–113.
- Xiang W & MacLaren LA 2002 Expression of fertilin and CD9 in bovine trophoblast and endometrium during implantation. *Biology of Reproduction* **66** 1790–1796.
- Xie S, Low BG, Nagel RJ, Beckers JF & Roberts RM 1994 A novel glycoprotein of the aspartic proteinase gene family expressed in bovine placental trophoctoderm. *Biology of Reproduction* **51** 1145–1153.
- Yadav PS, Kues WA, Herrmann D, Carnwath JW & Niemann H 2005 Bovine ICM derived cells express the Oct4 ortholog. *Molecular Reproduction and Development* **72** 182–190.
- Yamada O, Todoroki J, Kizaki K, Takahashi T, Imai K, Patel OV, Schuler LA & Hashizume K 2002 Expression of prolactin-related protein 1 at the fetomaternal interface during the implantation period in cows. *Reproduction* **124** 427–437.
- Yamaguchi TP, Bradley A, McMahon AP & Jones S 1999 A Wnt5a pathway underlines outgrowth of multiple structures in the vertebrate embryo. *Development* **126** 1211–1223.
- Yamamoto M, Saijoh Y, Perea-Gomez A, Shawlot W, Behringer RR, Ang SL, Hamada H & Meno C 2004 Nodal antagonists regulate formation of the anteroposterior axis of the mouse embryo. *Nature* **428** 387–392.
- Yates AJ & Rampersaud A 1998 Sphingolipids as receptor modulators. An overview. *Annals of the New York Academy of Sciences* **845** 57–71.
- Yelich JV, Pomp D & Geisert RD 1997a Detection of transcripts for retinoic acid receptors, retinol-binding protein, and transforming growth factors during rapid trophoblastic elongation in the porcine conceptus. *Biology of Reproduction* **57** 286–294.
- Yelich JV, Pomp D & Geisert RD 1997b Ontogeny of elongation and gene expression in the early developing porcine conceptus. *Biology of Reproduction* **57** 1256–1265.

Received 31 July 2007

First decision 11 September 2007

Revised manuscript received 12 November 2007

Accepted 21 November 2007