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REPRODUCTION

REVIEW

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Abstract

Preimplantation development directs the formation of an implantation- or attachment-competent embryo so that metabolic interactions with the uterus can occur, pregnancy can be initiated, and fetal development can be sustained. The preimplantation embryo exhibits a form of autonomous development fueled by products provided by the oocyte and also from activation of the embryo's genome. Despite this autonomy, the preimplantation embryo is highly influenced by factors in the external environment and in extreme situations, such as those presented by embryo culture or nuclear transfer, the ability of the embryo to adapt to the changing environmental conditions or chromatin to become reprogramed can exceed its own adaptive capacity resulting in aberrant embryonic development. Nuclear transfer or embryo culture-induced influences not only affect implantation and establishment of pregnancy but also can extend to fetal and postnatal development and affect susceptibility to disease in later life. It is therefore critical to define the basic program controlling preimplantation development, and also to utilize nuclear transfer and embryo culture models so that we may design healthier environments for preimplantation embryos to thrive in and also minimize the potential for negative consequences during pregnancy and post-gestational life. In addition, it is necessary to couple gene expression analysis with the investigation of gene function so that affects to gene expression can be fully understood. The purpose of this short review is to highlight our knowledge of the mechanisms controlling preimplantation development and report how those mechanisms may be influenced by nuclear transfer and embryo culture.
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Introduction

Preimplantation development directs the formation of an implantation- or attachment-competent embryo so that metabolic interactions with the uterus can occur, pregnancy can be initiated, and fetal development can be sustained (Watson 1992, Watson & Barcroft 2001). The preimplantation embryo exhibits a form of autonomous development fueled by products provided by the oocyte and also from activation of the embryo's genome (Schultz 2005). Despite this autonomy, the preimplantation embryo is highly influenced by factors in the external environment and in extreme situations, such as those presented by embryo culture or nuclear transfer, the ability of the embryo to adapt to changing environmental conditions or chromatin to become reprogramed can exceed its own adaptive capacity resulting in aberrant embryonic development (Niemann & Wrenzycki 2000, Schultz & Williams 2002, Gao *et al.* 2003). Nuclear transfer or culture-induced influences not only affect implantation and establishment of pregnancy but also can extend to fetal and postnatal development and affect susceptibility to disease in later life (Barker 2003, Ecker *et al.* 2004, Yang *et al.* 2007). It is therefore critical to define the basic program controlling preimplantation development, and also to utilize nuclear transfer and embryo culture models so that we may design healthier environments for preimplantation embryos to thrive in and also minimize the potential for negative consequences during pregnancy and post-gestational life. In addition, it is necessary to couple gene expression analysis with the investigation of gene function so that affects to gene expression can be fully understood. The purpose of this short review is to highlight our knowledge of the mechanisms controlling preimplantation development and report how those mechanisms may be influenced by nuclear transfer and embryo culture.

Q4 *Preimplantation development: blastocyst formation and embryonic genome activation (EGA)*

Preimplantation development is characterized by a series of cleavage divisions that subdivide the oocyte

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into smaller and smaller compartments, activation of the embryonic genome, compaction, cavitation (blastocyst formation), and finally zona hatching and implantation to the uterine wall (Watson 1992, Watson & Barcroft 2001). The principal achievement of preimplantation development is the formation of a fluid-filled structure called the blastocyst which is composed of an outer epithelial trophectoderm (TE), encircling a small group of cells called the inner cell mass (ICM) and a large fluid-filled cavity (Watson & Barcroft 2001). The TE, the first differentiated cell type of development, is a specialized tissue that initiates implantation or attachment and is the progenitor of the placenta. The ICM is the pluripotent progenitor of the embryo proper (Rossant 2004, Yamanaka *et al*. 2006). The program of preimplantation development is therefore directed at the formation of the TE and the specification of these distinct cell lineages. This process begins with the onset of compaction which also establishes cell polarity in the outer cells of the early embryo (Rossant 2004, Yamanaka *et al*. 2006). Compaction follows a major event in establishing the embryo's gene expression program called EGA. EGA has long been thought of as a global and promiscuous activation of genes whose regulated repression was then necessary to establish the preimplantation developmental program. Thanks to large-scale transcriptomic analyses, it now appears that a highly regulated gene expression program is initiated as soon as embryonic genome expression begins (Hamatani *et al*. 2004, Wang *et al*. 2004, Zeng *et al*. 2004). Because early perturbations of this 'embryonic program' have long-term significant effects on reproductive performance, we will discuss recent data concerning the regulation of EGA and its importance for long-term development.

Gene reprograming at embryonic genome activation: passage through totipotency

Reprograming reflects the ability of a nucleus to modify its gene expression pattern when placed in a new environment. Fertilization brings together with the haploid genomes of two highly differentiated cells, the gametes, into the oocyte cytoplasm. One of the first functions of the fertilized embryo is to reprogram the newly formed embryonic genome to a totipotent state. Totipotency is a rare and transient property, characterized by the ability of an individual embryonic cell to give rise to a whole, normal, and fertile individual. It is displayed only by fertilized eggs and early embryos in mammals, spans over few cell cycles, and is already lost at the blastocyst stage. Such reprograming relies on extensive epigenetic modifications of the genome that coordinates nucleo-cytoplasmic interactions. During fertilization gametic genomes are initially transcriptionally silent; gene reprograming is thus concomitant with embryonic genome transcriptional activation. Over the last decade, the interest in early embryonic genome reprograming has significantly increased with the awareness that mammalian oocyte cytoplasm is able to reprogram not only gametic genomes but also somatic cell genomes, although with a lower efficiency. Both genetic alterations of the oocyte cytoplasmic content and nuclear transfer experiments, primarily applied to mice and cows have provided new approaches to understand this unique property of the oocyte. While they are compatible with high rates of preimplantation development, these manipulations induce long-term effects; only a small percentage of the somatic cell nuclear transfer embryos develop to birth, for example, and depletion of the oocyte cytoplasm in maternal Ezh2, involved in epigenetic remodeling of the embryonic genome provokes reduction of birth weight in mice (Erhardt *et al*. 2003). These long-term developmental effects are attributed to faults in genome reprograming pointing to the crucial role of early epigenetic events for long-term development.

Early development relies on maternal transcripts and proteins that are progressively degraded while embryonic genome transcription progressively increases. This maternal to embryonic transition (MET) provides the embryo with the opportunity to restrict the maternally encoded genetic program and to set up an embryonic program of gene expression (Schultz 2005). It has been now extensively characterized by large-scale transcriptomic analyses in mice, where three groups of genes have been identified: genes encoding oocyte-specific transcripts that are definitively eliminated during MET, genes encoding embryonic transcripts whose expression begins at EGA, and genes whose transcripts are first inherited from the oocyte then synthesized from the embryonic genome (representing only about 40% of the genes; Hamatani *et al*. 2004). The first two categories of genes are responsible for the global change in the program of gene expression during the period of EGA. In the case of fertilization, reprograming at EGA thus corresponds to both a change in the genetic origin of the transcripts (maternal or embryonic) and a change in the program of gene expression; it occurs without any significant change in embryo morphology. Functional changes are in fact more progressive in the embryo; both the rates of maternal transcript degradation and the stability of the maternally encoded proteins vary so that some maternal information may still contribute to embryonic development after EGA.

EGA in the mouse embryo

In the mouse embryo, EGA occurs at the two-cell stage (although transcription is first detected in the male pronucleus prior to pronuclear fusion), that is, early during the cleavage period and long before the first differentiation at the blastocyst stage (Schultz 2005). Among genes transcribed at EGA, genes involved in basic cellular function, ion transport, ribonucleotide

metabolism, and also ribosome biogenesis, protein synthesis, RNA metabolism, and transcription are over-represented (Hamatani *et al*. 2004, 2006, Zeng & Schultz 2005). A second transition in gene expression has been reported between the four- and eight-cell stages in the mouse, corresponding to the activation of genes which may be key regulators of TE differentiation (Hamatani *et al*. 2004). A subgroup of genes are transiently expressed at each cleavage stage (Hamatani *et al*. 2004, Zeng *et al*. 2004). In particular, expression of Q6 LTR retrotransposons is reported to occur at EGA (Evsikov *et al*. 2004), and specific transposable elements act at that stage as alternative promoters and first exons for a subset of host genes transcribed as chimeric transcripts (Peaston *et al*. 2004). The expression of such repetitive elements may be regulated by RNA interference mechanisms (Svoboda *et al*. 2004).

In mice, EGA is concomitant with extensive epigenetic remodeling of the parental genomes into the newly formed embryo (Morgan *et al*. 2005). Epigenesis involves all the factors modifying gene expression in a cell-division heritable way, without any alteration of DNA sequence (Holliday 1994). It is responsible for the acquisition of different gene expression programs in different cells during the development of multicellular organisms. Epigenetic marks involve posttranslational modifications (methylation, acetylation, phosphorylation, and ubiquitination) of nucleosomal histones, DNA methylation, and non-histone proteins that bind to chromatin. Briefly, transcriptionally inactive heterochromatin is characterized by deacetylated histones, methylation of histone H3 lysine 9, and DNA methylation, whereas acetylation of H3 and H4 histones, methylation of histone H3 lysine 4, and low level of DNA methylation are associated with active euchromatin regions. These modifications of nucleosomal histones alter the higher-order chromatin structure to render the DNA accessible to the regulatory and transcriptional machinery. These different levels of epigenetic marks tightly interact: proteins displaying high affinity for methylated DNA, for example, associate with histone deacetylase and methyltransferase.

In the mouse, at fertilization, the metaphase 2 arrested maternal genome is packaged with histones already displaying various modifications (acetylation or methylation) in different regions of the genome and exhibits a relatively high level of DNA methylation. In the paternal genome, protamines are first replaced with histones which are more acetylated than those inherited by the maternal genome but evidence of early histone methylation appears soon after this incorporation. An active demethylation of the paternal DNA then occurs before DNA replication and only some specific regions of Q6 heterochromatin around centromeres, IAP retrotransposons, and paternally methylated imprinted genes escape it (Morgan *et al*. 2005). Both parental genomes are thus epigenetically asymmetric which is likely responsible for the precocious transcriptional activation of the paternal genome observed in the mouse. During the first cleavages, a passive DNA demethylation of the whole embryonic genome progressively occurs due to maternally inherited Dnmt1 exclusion from the nuclei, resulting in a low methylation level at the morula stage (Morgan *et al*. 2005). Whether histone modifications are also reprogramed during this passive phase of DNA demethylation remains unclear. Later on, differential *de novo* remethylation occurs in the ICM due to preferential localization of Dnmt3b in these cells rather than TE cells.

Is the mouse embryo a representative model for EGA reprograming?

Neither the abrupt kinetics of EGA occurring at the two-cell stage nor the extent of associated epigenetic remodeling events are shared by non-murine embryos. In all non-murine embryos, EGA spans over several cell cycles with a weak transcriptional activity from the end of the 1-cell stage, but a major transcriptional activation at the 4- (pig, human) or 8- to 16-cell stage (sheep, bovine, rabbit; Telford *et al*. 1990). This implies a longer reliance on maternally inherited information and a shortened delay between EGA and cell differentiation. Whether this affects the nature of genes preferentially expressed at EGA, or the number of transcription waves, remains unknown since very few large-scale analyses of gene reprograming at EGA have been published in these species (Whitworth *et al*. 2004, Misirlioglu *et al*. 2006). In cattle, early genes that are transcribed include genes involved in transcription regulation, cell adhesion, signal transduction, transporters, and metabolism (Misirlioglu *et al*. 2006). Transient expression of genes at EGA has also been reported in the rabbit (Pacheco-Trigon *et al*. 2002) but large-scale comparisons of early encoded functions in species with different EGA kinetics remain to be done.

In addition, the extent of epigenetic changes associated with EGA varies between species (Beaujean *et al*. 2004). DNA methylation has been mainly investigated. Active paternal DNA demethylation is less pronounced in cattle than in the mouse, it is undetectable in sheep and rabbit, and a partial asymmetrical demethylation has been reported in only half of human embryos (Fulka *et al*. 2004). Subsequent passive demethylation also differs among species, being reduced in sheep and barely detectable in the rabbit. The differential remethylation of the ICM also varies since both in the rabbit (Shi *et al*. 2004) and in the human (Fulka *et al*. 2004), DNA methylation is higher in the TE than in ICM. Variation in levels of histone deacetylases and histone acetyltransferases throughout bovine embryonic development have been reported (McGraw *et al*. 2003, 2007). McGraw *et al*. (2003, 2007) described the temporal expression profile, during preimplantation embryo development, of

15 key regulators involved in RNA, DNA or histone methylation, chromatin modification or silencing, and transcription regulation; all were present to different degrees in the developmental stages tested, and they can be divided into three different groups depending on their respective mRNA profile. More detailed comparative analysis of specific regions of the genome have yet to be carried out in order to understand the developmental consequences of these epigenetic reprograming events, their consequences on gene expression reprograming at EGA and on totipotency reprograming.

Long-term consequences of reprograming at EGA: somatic cell nuclear transfer embryos as an experimental model

Although the birth of normal, fertile cloned animals from many species proves that a sufficient reprograming may be obtained after somatic cell nuclear transfer (SCNT), long-term developmental effects likely related to early reprograming faults frequently occur (Yang *et al*. 2007). Large variations in full-term developmental potential of various donor cell types are now well described but still not understood (Panarace *et al*. 2007). Molecular studies are aimed at characterizing the extent of reprograming faults and their functional consequences. Reprograming here refers to both extinction of genes expressed by the differentiated donor cell, and transcriptional activation of embryonically expressed genes, which includes extensive epigenetic reprograming. Comparing early SCNT embryos to control embryos revealed their abnormal epigenetic status. In cattle, for example, passive demethylation is delayed and weakened in SCNT embryos; in addition, histone acetylation and methylation patterns are altered (Santos *et al*. 2003, Beaujean *et al*. 2004). Faults in gene expression are also observed at various developmental stages. During the preimplantation period in the mouse, alterations in imprinted gene expression (Mann *et al*. 2004), persistent expression of genes specific to the donor nucleus (Gao *et al*. 2003), and deficient expression of genes involved in pluripotency maintenance (Boiani *et al*. 2002) are reported. These candidate gene analyses remain, however, unsatisfactory to understand reprograming faults since results highly depend on the gene, the stage of analysis, the technique used for nuclear transfer, and the donor cell type.

Resorting large-scale transcriptional studies should help to determine general trends of early reprograming and to identify relevant candidate genes, if any. Most studies have reported that a global reprograming has already occurred by the blastocyst stage since the SCNT blastocyst gene expression program is closer to that of control blastocysts than to that of the donor cell (Pfister-Genskow *et al*. 2005, Smith *et al*. 2005, Beyhan *et al*. 2007). This reprograming appears, however, incomplete since some genes are differentially expressed between control and SCNT blastocysts. At that stage, however, the relationship between gene expression profiles and embryo full-term development is probably not direct (Smith *et al*. 2005). Tracking earlier gene reprograming faults should provide information about initial events. Global reprograming already occurred in cattle SCNT morulae (Duranthon *et al*. unpublished results) and genes involved in transcription and its regulation are mis-regulated at EGA in mouse cloned embryos, probably leading to further gene expression abnormalities (Vassena *et al*. 2007). Further analyses are required to better integrate such early events and their long-term consequences and these should also take into account interspecific variations.

Preimplantation development: from totipotency to the first differentiative events

Compaction and cell polarity

The first step toward differentiation is to establish intercellular communication. Compaction is signaled by an increase in cell-to-cell contact between embryonic blastomeres. It is driven by the establishment of adherens junctions consisting of E-cadherin and catenin complexes (Fleming *et al*. 2000, Johnson & McConnell 2004). Compaction is initiated at the eight-cell stage in the mouse but the timing varies across species (Telford *et al*. 1990). In all cases, it results in the formation of a morula by which the 16-cell stage in the mouse creates a topology that forms outer and inner cells that are completely surrounded by the outer cells (Fleming *et al*. 2000, Johnson & McConnell 2004). These cell types are the progenitors of the TE and ICM.

Trophectoderm differentiation

The predominant models of TE differentiation include the 'inside–outside hypothesis' and 'the cell polarity model' (Tarkowski & Wroblewska 1967, Johnson & Ziomek 1981*a*,1981*b*; Fig. 1). The inside–outside hypothesis states that lineage specification is defined by position and cell-to-cell contact so that inner cells are subject to symmetrical contact, while outer cells maintain contact on three sides and have a free apical surface which defines a polarity axis as reflected by formation of focal tight junctions and adherens junctions (Johnson & Ziomek 1981*a*,1981*b*, Yamanaka *et al*. 2006). The cell polarity model predicts that cell fate is established at the eight-cell stage in the mouse and propagated by symmetrical or asymmetrical cell divisions that either generate two polar cells by dividing a radial polarity axis or an outer polar and an inner apolar cell (Johnson & Ziomek 1981*a*,1981*b*, Yamanaka *et al*. 2006). Although very similar, the models differ by suggesting that cell position directs cell fate (inside–outside) versus cell fate driving cell position (polarity model). Studies appear to favor the cell polarity model and have recorded polarized lectin binding, apical microvilli, cytoskeletal

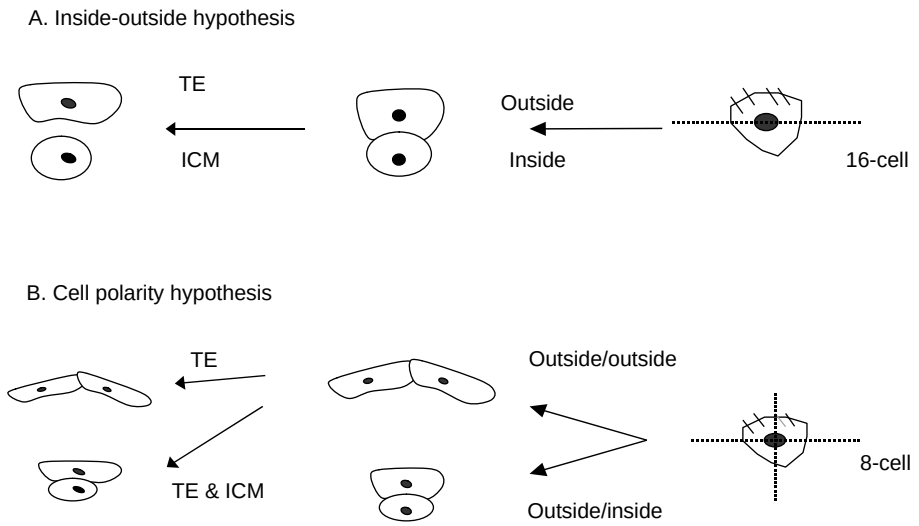

Figure 1 Cell polarization models during compaction. (A) The inside–outside hypothesis states that lineage specification is defined by position and cell-to-cell contact so that inner cells are subject to symmetrical contact while outer cells maintain contact on three sides and have a free apical surface which defines a polarity axis as reflected by formation of focal tight junctions and adherens junctions. (B) The cell polarity model states that cell fate is established at the eight-cell stage and propagated by symmetrical or asymmetrical cell divisions that either generate two polar cells by dividing a radial polarity axis or an outer polar and an inner apolar cell. Although very similar the models differ by suggesting cell position directs cell fate (inside–outside) versus cell fate driving cell position (polarity model; Johnson & McConnell 2004).

elements, and other organelles in eight-cell stage mouse embryos (Johnson & McConnell 2004, Yamanaka *et al*. 2006). In addition, studies have indicated that while polarity is established more efficiently in the presence of cell-to-cell adhesion, it is not required to maintain polarity once it has been established. Finally, polarization and compaction both occur in the presence of protein synthetic inhibitors indicating that these events are driven by posttranslational processes applied to a preexisting protein pool (Kidder & McLachlin 1985, Wiley *et al*. 1990). What are the key proteins directing polarity and compaction? Adherens junction components are among the most critical proteins involved in the establishment of polarity and compaction but Q6 others include the PAR complex (PAR 1, 3, and 6), atypical PKCs, and tight junction-associated proteins (Yamanaka *et al*. 2006). Since these proteins contribute to the formation of an apical protein complex their position satisfies the conditions of the cell polarity model. Thus, the foundation for TE formation lies in compaction and establishment of cell polarity in outer embryonic blastomeres. But which factors control the decision to become TE or ICM? Recent research applied to the mouse has established that TE and ICM differentially express several lineage-specific transcrip- Q6 tion factors. Cdx2 becomes restricted to the TE and is required for TE formation (Yamanaka *et al*. 2006; Fig. 2). In contrast, Oct4 and Nanog become restricted to and influence ICM fate (Yamanaka *et al*. 2006; Fig. 2). The current understanding of their roles has lead to a model Q6 that predicts mutual antagonism between Oct4 and Cdx2 in supporting the formation of TE and ICM fates in the blastocyst (Yamanaka *et al*. 2006). It will be interesting to see whether this model extends to other mammalian species.

Blastocyst formation

Blastocyst formation or cavitation is dependent upon TE differentiation as it is the ion and water transport functions of the TE that mediates the fluid dynamics that control blastocyst formation (Watson 1992, Watson & Barcroft 2001). Na/K-ATPase, aquaporins (AQP; water channels), and tight junctions have established roles in

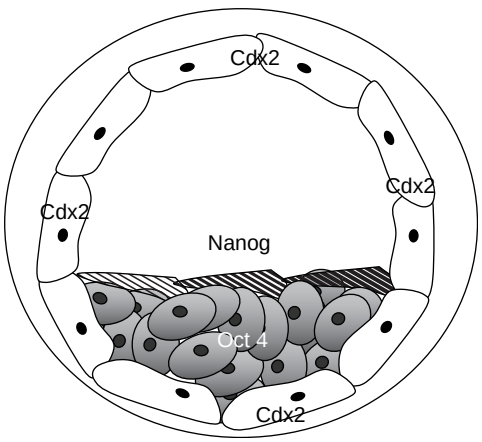

Figure 2 Cell lineage and transcription factors. Trophectoderm and inner cell mass differentially express lineage-specific transcription factors. Cdx2 becomes restricted to the trophectoderm and is required for trophectoderm formation. In contrast, Oct4 and Nanog become restricted to and control inner cell mass fate. The model predicts mutual antagonism between Oct4 and Cdx2 in supporting the formation of trophectoderm and inner cell mass fates in the blastocyst (Yamanaka *et al*. 2006).

coordinating blastocyst formation (Watson 1992, Watson & Barcroft 2001). The model that has been tested is that blastocyst formation is dependent upon the polarized distribution of the Na/K-ATPase confined to the basolateral membrane domains of the TE. This establishes a trans-trophectoderm ion gradient that facilitates movement of water across the epithelium facilitated by the presence of both apical and basolateral AQPs (Fig. 3). These events combined with the establishment of a TE tight junctional seal to prevent the loss of fluid out of the embryo through paracellular routes results in the expansion of the embryo and the formation of the blastocyst (Watson 1992, Watson & Barcroft 2001). Over the years, considerable evidence has been collected that supports this hypothesis.

Na/K-ATPase is confined to the basolateral membrane domain of the mural TE (Watson & Barcroft 2001). In addition, enzyme activity increases just prior to blastocyst formation in all mammalian species examined to date (Watson 1992, Watson & Barcroft 2001). The expression of all the principal Na/K-ATPase isozymes has been defined as well as the functions of Na/K-ATPase α1 and β1 subunits in supporting blastocyst formation in the mouse (Watson *et al*. 1990, MacPhee *et al*. 2000, Barcroft *et al*. 2004, Madan *et al*. 2007). In addition, aquaporins and their role in facilitating blastocyst formation in the mouse have been investigated (Barcroft *et al*. 2003, Offenberg & Thomsen 2005). Treatment of mouse blastocysts with pCMPs (mercuric AQP blocker) results in the attenuation of the fluid transport that accompanies exposure of mouse blastocysts to hyperosmotic media (Barcroft *et al*. 2003; Fig. 3). In addition to these critical gene products, recent studies have applied subtractive hybridization and gene array screening methods to identify a growing list on genes that are implicated in regulating compaction and blastocyst formation (Ko *et al*. 2000, Hamatani *et al*. 2006, Goossens *et al*. 2007). These studies are invaluable for identifying and directing studies to new gene targets that are implicated in the basic program that governs preimplantation development. They ensure that our understanding of the basic program controlling preimplantation development will remain a very rich area of research well into the future.

What is next for these studies? Over the past few years, four different endogenous cardiotonic steroids (CTS) have been isolated from human plasma, bovine adrenals, hypothalamus, and amniotic fluid. These compounds include ouabain (identical to the plant-derived steroid), digoxin, marinobufagenin, and 19-nobufalin (Schoner 2002). The adrenal is the likely source of their production as levels decline dramatically following adrenalectomy in dogs and bovine adrenal-cortical cells secrete high levels of ouabain *in vitro* (Schoner 2002). Research is just beginning to define their physiological roles. The presence of these compounds in plasma is certain although their appearance in the reproductive tract has not been determined. They may, however, represent a novel hormonal signaling pathway for regulating blastocyst formation *in vivo*. In addition, one of the most exciting discoveries in recent years is that following cardiotonic steroid binding to the Na/K-ATPase c-SRC tyrosine kinase forms a binary receptor which phosphorylates and assembles additional proteins into signaling modules which activates MAP kinase pathways (MAPK) and protein kinase C isozymes in a cell-specific way. We therefore speculate that in addition to its better recognized role of regulating ion transport, the Na/K-ATPase is also an important signaling molecule that activates c-SRC-mediated MAPK pathways that regulate cell junction formation during preimplantation development. The future will tell.

Impact of culture on preimplantation development

In addition to nuclear transfer, measuring the influences of culture on embryonic gene expression has emerged as an important experimental paradigm for investigating

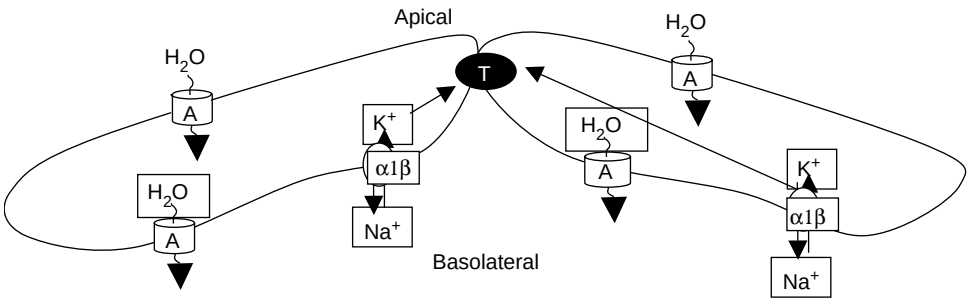

Figure 3 Blastocyst formation model. Blastocyst formation is dependent upon trophectoderm differentiation as the ion and water transport functions of the trophectoderm mediate the fluid dynamics that control blastocyst formation. The hypothesis we have tested is that blastocyst formation is dependent upon the polarized distribution of the Na/K-ATPase confined to the basolateral membrane domains of the trophectoderm. This establishes a trans-trophectoderm ion gradient that facilitates movement of water across the epithelium facilitated by the presence of both apical and basolateral AQPs. These events combined with the establishment of a trophectoderm tight junctional seal to prevent the loss of fluid out of the embryo through paracellular routes results in the expansion of the embryo and the formation of the blastocyst (Watson & Barcroft 2001).

epigenetic and environmental influences on preimplantation development and their longer terms effects on fetal and *post partum* development. We have presented that precise control of gene expression during preimplantation development is particularly important as several developmental events occur during this period including: i) the first mitotic division, the timing of which has been associated with developmental competence in a variety of *mammalian* species (Lonergan *et al*. 1999, 2006), ii) embryonic genome activation, when the embryo transfers from a reliance on maternal RNA derived from the oocyte to expression of its own genome (Telford *et al*. 1990), iii) morula compaction, and iv) blastocyst formation, as described above, and in ruminants, subsequent elongation prior to implantation.

While few studies have examined the temporal pattern of transcript abundance from zygote to blastocyst stage, the large majority of reports describe relative transcript abundance at the blastocyst stage only. While formation of the blastocyst is undoubtedly an important checkpoint/landmark on the developmental axis, it is important to remember that the blastocyst is the product of a sequence of events which precede it, as outlined above. Although not the main subject of this review, there is evidence to demonstrate that the environment to which the oocyte is exposed during maturation can influence the pattern of transcripts in the matured oocyte (Watson *et al*. 2000, Lonergan *et al*. 2003) and in the resulting blastocyst (Russell *et al*. 2006). However, most evidence suggests that the pattern of mRNA abundance in the blastocyst, and the quality of the blastocyst in terms of establishing and maintaining a pregnancy, is dictated by the post-fertilization conditions of culture (Lonergan *et al*. 2006). For example, Knijn *et al*. (2002) examined transcript abundance in cattle blastocysts derived from oocytes matured either *in vitro* or *in vivo* and found no differences for the small panel of transcripts examined, suggesting that blastocysts produced in a common post-fertilization culture environment have a similar transcript profile irrespective of the origin of the oocyte. In addition, several groups have reported that culture of *in vitro* produced zygotes *in vivo* in the sheep (Rizos *et al*. 2002) or cow (Tesfaye *et al*. 2007) oviduct results in embryos with a morphology, pattern of mRNA expression and an ability to withstand cryopreservation, similar to that of true *in vivo* derived embryos. There is a large and continually increasing body of evidence demonstrating that the culture environment to which embryos are exposed *in vitro* can perturb gene expression in the developing embryo. While this applies mainly to the culture medium used and its inclusions (Wrenzycki *et al*. 2005, Lonergan *et al*. 2006), the conditions of incubation are also important; for example, the relative abundance of specific transcripts in cow *in vitro* produced embryos alters in response to changes in the oxygen environment post-compaction (Harvey *et al*. 2004).

***Culture of IVP zygotes* in vivo**

Heterologous versus homologous culture

The oviductal environment can support embryonic growth up to the blastocyst stage across a wide range of species following trans-species transfer (Rizos *et al*. 2007). Culture of cow embryos in the oviduct of the ewe is suitable for the development of embryos from the zygote to blastocyst stage and even through the early stages of elongation. Though not perfect, one advantage of this *in vivo* culture system is the ability to culture large numbers of embryos in a 'near *in vivo*' environment and in a cost-effective manner. While the yield of blastocysts following such *in vivo* culture is not superior to that following culture *in vitro*, the quality of the blastocysts is significantly improved (Rizos *et al*. 2002). However, heterologous transfer and culture of embryos is never totally satisfactory from an experimental design viewpoint. Recently, endoscopy has been successfully used to access the oviducts of cattle for the *in vivo* culture of *in vitro* matured or fertilized embryos in the homologous oviduct (Besenfelder *et al*. 2001, Tesfaye *et al*. 2007). While this technique requires a significant level of skill and experience (currently only practiced routinely by one group worldwide for the tubal transfer of embryos) it offers much promise for comparative studies of embryo development and gene expression *in vivo* and *in vitro*.

Effect of in vivo embryo environment on embryo gene expression

Ruminants experience relatively high rates of embryonic and early fetal mortality (about 40%). Published estimates indicate a fertilization rate of 90% and an average calving rate of about 55%, suggesting an embryonic/fetal mortality of about 35%; it is estimated that 70–80% of the total embryonic loss occurs between days 8 and 16 after insemination (day 16 corresponding to the period of maternal recognition of pregnancy; Sreenan *et al*. 1999). The importance of progesterone in the establishment and maintenance of pregnancy in ruminants is well-known. While there is much evidence showing the importance of progesterone levels in the immediate postconception period (days 4–7) on subsequent pregnancy maintenance (McNeill *et al*. 2006) and increasing data on progesterone-induced changes in gene expression in the uterus (Spencer *et al*. 2004), there is little known about the gene expression changes induced in the embryo at this time. Advancement of conceptus development following administration of early exogenous progesterone has been described in both cattle (Garrett *et al*. 1988) and sheep (Satterfield *et al*. 2006). Despite the presence of mRNA for the progesterone receptor on cow embryos (Fair *et al*. unpublished), evidence for a direct effect of progesterone on embryo development is lacking. Addition of progesterone to culture medium is reported to have no effect

or only to have an effect in the presence of coculture cells (Lavranos & Seamark 1989). The mechanisms through which preimplantation concentrations of progesterone regulate embryo survival and growth are not well investigated but are thought to be mediated by secretions from the endometrium. In sheep, progesterone acts on the endometrium to induce a number of genes that encode for proteins secreted into the uterine lumen, including galectin 15 (LGALS15) and secreted phosphoprotein one or (osteopontin). The advanced development of blastocysts in progesterone-treated ewes is hypothesized to involve early induction of specific genes in the endometrial epithelia, such as LGALS15 and components of uterine histotroph (Satterfield *et al*. 2006) and it is likely that a similar mechanism operates in cattle although the precise details have not yet been elucidated.

Understanding the implications of culture-induced changes in mRNA abundance

Just what does it mean for the embryo when the relative abundance of certain transcripts alters in response to a changing environment? The consequences of differential mRNA abundance for subsequent development (i.e., the functional significance of such changes) are difficult to interpret. Few studies have attempted to correlate the differences in mRNA abundance observed at the blastocyst stage, such as those outlined above, with the ability of the embryo to establish a pregnancy. One such study (El-Sayed *et al*. 2006) addressed the relationship between transcriptional profile of embryos and the pregnancy success based on gene expression analysis of blastocyst biopsies taken prior to transfer to recipients. Microarray data analysis revealed a total of 52 differentially regulated genes between embryos resulting in a calf delivery versus those not resulting in a pregnancy. Biopsies resulting in calf delivery were Q6 enriched with genes necessary for implantation (COX2, Q6 CDX2), carbohydrate metabolism (ALOX15), growth Q6 factor (BMP15), signal transduction (PLAU), and placental development (PLAC8), while those failing to establish a pregnancy were enriched with transcripts Q8 for inflammatory cytokines tumour necrosis factor (TNF), Q6 protein amino acid binding (EEF1A1), transcription Q6 factors (MSX1, PTTG1), glucose metabolism (PGK1, Q6 AKR1B1), and inhibition of implantation (CD9). Another approach toward testing functionality is to examine genes found to be differentially expressed in one model in a second model of competence (Mourot *et al*. 2006, Patel *et al*. 2007). A more direct approach is to alter the levels of mRNA (either under express or overexpress) and look for a phenotypic effect. RNA interference (RNAi) has become a well-established technique to study gene function in several species (Madan *et al*. 2007). In domestic species, however, the use of RNA interference technology in domestic species is still in its infancy with only a handful of papers published on the subject (Tesfaye *et al*. 2007). It will be critical to define gene function so that a full interpretation of culture-induced changes in gene expression can be arrived at.

Concluding statements

Preimplantation development is characterized by extensive epigenetic modifications of the newly formed embryonic genome that permits the onset of a highly regulated gene expression program. While this program is directed at the formation of a functional implantation-competent blastocyst, alterations of its initial steps affect embryo development far beyond this stage. Current research is focused upon understanding the epigenetic mechanisms that enable the early embryo to initiate its normal developmental program and also adjust that program to respond to environmental perturbations. Paradoxically the ability to support early embryo development *in vitro*, which of course has been of great benefit to understanding the mechanisms controlling early development and to also providing new ways to propagate animal species and treat human infertility, has now come under greater scrutiny due to its capacity to affect the embryonic developmental program and thus influence fetal and *post partum* development. To alleviate these concerns it is vital that research focus once again on *in vivo* development. The functional analysis of the molecular basis of these culture or nuclear transfer-based alterations will reveal new aspects of early developmental regulation. It should help to better define the limits of the early mammalian embryo developmental autonomy and is necessary to design healthier *in vitro* conditions, and to perhaps the development of new strategies to modify individual phenotypes.

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