Gene expression in elongating and gastrulating embryos from ruminants

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In ruminants, more than 30% of the embryonic losses observed after artificial insemination (AI) have an early origin, coincident with a marked elongation of the trophoblast which occurs before implantation. Several observations provide clear evidence that early elongation of the conceptus relies on cell multiplication, cell growth and cell shape remodeling. Recent results indicating an intense multiplication of a non-fully differentiated trophoblast, which still expresses some epiblast genes, has to be considered at the onset of elongation. It has also been shown in the last two years that general metabolism and protein trafficking are characteristic of the onset of elongation whereas cellular interactions, cell to cell signaling and cell adhesion become predominant at the end of elongation. Accordingly, expression of most of the single genes identified so far increases during elongation and is related to the establishment of embryo-maternal exchanges before implantation. However, not much is known of what controls the induction of the elongation process or the coordinated development of the embryonic and extra-embryonic tissues. This review highlights new information on this developmental phase and summarizes the views on the complex cross-talk among molecules which might govern conceptus development and lead to successful implantation.

Introduction

After fertilisation and cleavage, the mammalian embryo reaches the blastocyst stage. This stage occurs after *in vivo* or *in vitro* development and is characterised by the morphological distinction between two cell types: the inner cell mass (or ICM) and the trophectoderm, even though molecular distinctions between these lineages appear earlier (Rossant 2004). In ruminants, the blastocyst is a sphere of 150-200 μm in diameter which contains approximately 250-300 cells (8 days after mating or insemination). A few days later, the sphere has enlarged, the number of cells is 10 times higher and the blastocyst has evolved to an ovoid shape (Day 11 in sheep, Day 12 in cows). According to Chang (1952) the ICM evolves into a germinal disc and the endoderm cells underlying the disc migrate under the trophectoderm to form an extra-embryonic endoderm layer. On Day 12 in sheep and Day 14 in cows, the blastocyst has a tubular shape, and the germinal disc is a true embryonic disc that is flat and open to the uterine environment since the trophoblast layer (called polar trophoblast or Rauber's layer) which covers the ICM

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until this stage has disappeared. Subsequently, the mural trophoblast lineage further elongates and gives rise to a filamentous-shaped conceptus (Day 14 in sheep, Day 16 to 18 in cows) composed of differentiated embryonic and extra-embryonic tissues. The extra-embryonic tissues include the chorion, formed by fusion of trophectoderm with migrating extra-embryonic mesoderm, and the growing yolk sac composed of extra-embryonic endoderm and mesoderm. Growth and differentiation processes of embryonic and extra-embryonic lineages are referred to as gastrulation and elongation, respectively. Finally, the trophoblast forms loose cellular contacts with the endometrial layer of the uterus, which marks the onset of implantation (Day 15 in sheep, Day 19 in cows; Guillomot 1995).

This review focuses on the process of conceptus elongation without comments on trophoblast lineage differentiation or implantation which were reviewed recently (Roberts *et al.* 2004; Spencer & Bazer 2004). We will concentrate on recent molecular data and comment on elongation models to gain insights into this complex process, mostly studied as an essential step in establishing functional embryo-maternal communications (Wolf *et al.* 2003; Imakawa *et al.* 2004; Klein *et al.* 2006). Maternal recognition of pregnancy is indeed essential for the uterus to switch from a cyclic to a pregnant state as underlined by the high rate of embryonic loss before implantation (Anderson 1978; Dunne *et al.* 2000; Goff 2002). This review will mainly describe gene expression patterns in ruminants, but occasionally comments on results obtained from studies of porcine conceptuses which also elongate and gastrulate before implantation.

Elongation and extra-embryonic development

Elongation defines the exponential growth of bovine, ovine and porcine blastocyst which occurs before implantation, with rapid evolution in their shapes from spherical to ovoid, tubular and finally filamentous forms (see Fig. 1). Though simple in its description, elongation is a complex process which does not have a clear starting point. Depending on species, elongation starts when conceptuses are at a spherical to slightly ovoid stage (pig) or an ovoid to slightly tubular stage (sheep, cow), on Day 11 (pig, sheep) or Day 12 (cow) post-insemination and at diverging blastocyst sizes: 1 to 2 mm (sheep, cow) or 9 to 10 mm (pig). It seems that these parameters (Wintenberger-Torres & Flechon 1974; Geisert *et al.* 1982; Betteridge & Flechon 1988), though extremely useful when collecting the embryos, are not adequate to establish a clear starting point to understand it at the molecular level. Therefore, we define the 150-200 μm freshly hatched blastocyst as the developmental stage for beginning elongation, the spherical-ovoid transition as the induction phase of elongation, the tubular-filamentous transition as a step of elongation maintenance and implanting stages as a phase of elongation arrest.

When starting elongation at the early blastocyst stage, cell proliferation likely constitutes the initiating event of the process. The clear increase in cell numbers initially observed between spherical and ovoid stages (Chang 1952; Wintenberger-Torres & Flechon 1974) has been confirmed using several molecular approaches. Analysing the transition in development of bovine blastocysts between Days 7 and 14, Ushizawa *et al.* (2004) identified 680 genes for which expression increased and 26 genes that were down-regulated including those encoding for oncogenes, tumor suppression, cell cycle control and apoptosis (see Fig. 1). Similarly, cell cycle and cell proliferation related genes represented 29% of the annotated repertoire we established at the ovoid stage (Degrelle *et al.* 2005) while a small set of genes preferentially expressed at the ovoid stage included a marker of cell proliferation in human cancers (Nap1L1). However, since none of these transcripts has been localised *in situ* at spherical or ovoid stages, the molecular and cellular basis of this proliferation process remains poorly characterised.

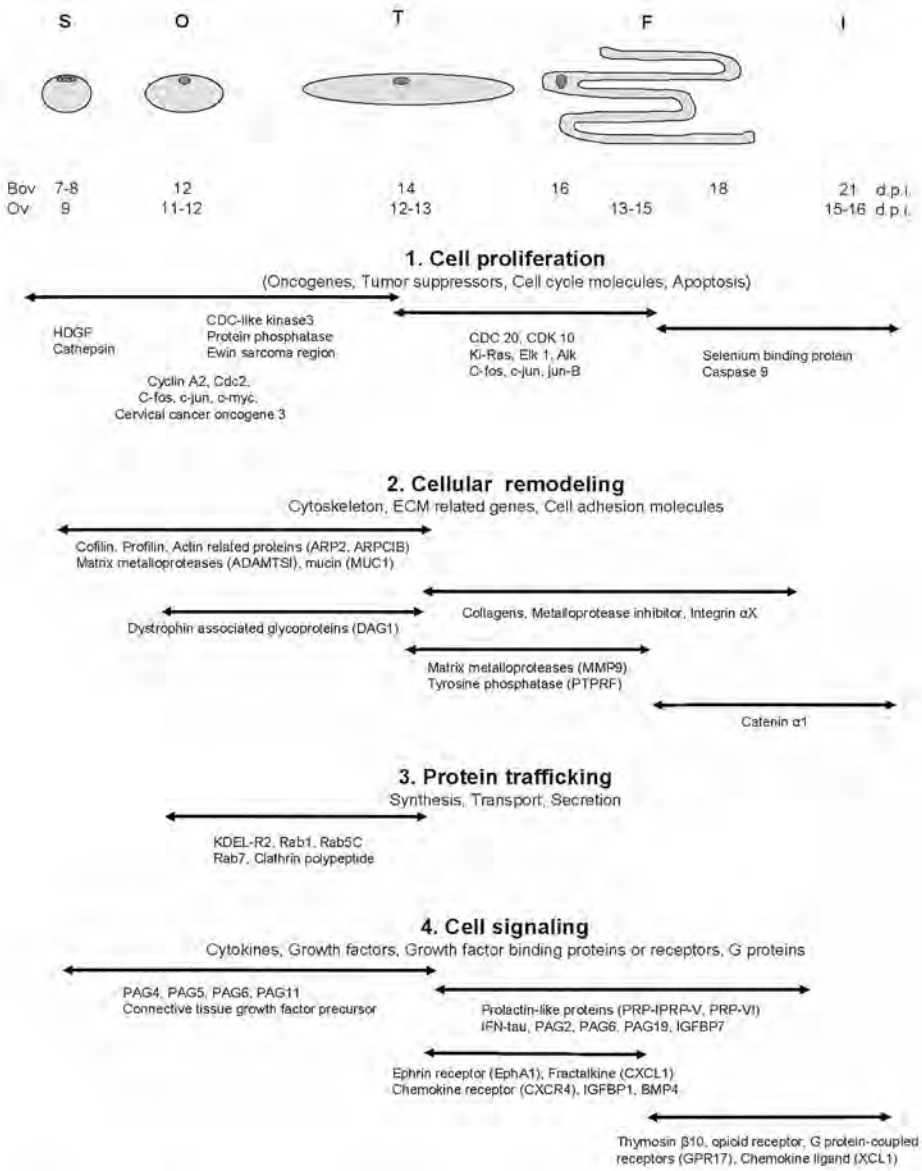

Fig. 1. Gene expression across elongation in ruminants. Some of the genes known to be expressed across elongation are named here together with the functions they relate to. Differential expression patterns are symbolised by horizontal arrows encompassing the appropriate stages: spherical (S), ovoid (O), tubular (T), filamentous (F) or implanted (I). These sets of genes originate from the following reports: Ushizawa *et al.* 2004; Cammas *et al.* 2005; Degrelle *et al.* 2005; Xavier *et al.* 1997; ovoid cDNA library (I Hue & SA Degrelle, unpublished observations). The conceptuses are schematically drawn according to their shape: light grey represents extra-embryonic tissues, dark grey: embryonic tissues. The developmental timing is scaled in days post insemination (or d.p.i) where day 0 marks the onset of oestrus.

In addition to cell proliferation, the onset of elongation involves a cellular reorganisation based upon changes in cell morphology, density or migration. At the spherical stage, the trophectoderm is a layer of flattened cells and the endoderm cells which migrate out from the inner cell mass, start underlining the trophectoderm layer to give rise to the extra-embryonic endoderm. At the ovoid stage, these endoderm cells are flattened too, except at the level of the nucleus, whereas the trophectoderm cells are cuboidal and show numerous signs of phagocytosis (Betteridge & Flechon 1988). In the pig, changes in the cellular organisation of both trophectoderm and endoderm layers have been put forward to explain the quick transitions observed in the size and shape of elongating conceptuses. Indeed, within a thin band of cells called "elongation zone", trophectoderm cells exhibited alterations of microfilaments and junctional complexes whereas endoderm cells formed numerous filopodia (Geisert *et al.* 1982). No similar observations of an "elongation zone" (surrounding the ICM and extending from the disc to distal ends of the conceptus) have been reported for ruminants but genes related to cell migration and cell remodelling have been identified in bovine and ovine conceptuses (Fig. 1). Similarly, genes involved in protein trafficking (which includes synthesis, secretion and transport) were highly expressed in sheep as previously revealed on transmission electron micrographs by the presence of rough endoplasmic reticulum or endocytic vesicles (Betteridge & Flechon 1988). Cell differentiation and remodelling have also been explored at "late" stages of elongation and associated with implantation (Guillomot 1995; Spencer *et al.* 2004; Ushizawa *et al.* 2004; Cammas *et al.* 2005).

Last but not least, the elongation process has also been defined as an essential step in the establishment of pregnancy through an active cross-talk between the conceptus and the uterus well ahead of implantation. Cell signalling molecules have thus been identified accordingly (Fig. 1) and reported as increasingly expressed throughout the whole process or preferentially expressed at "late" elongated stages.

Despite the obvious roles of cell proliferation, cellular remodelling and cell signalling in the elongation process, most of the genes related to those functions were reported as "expressed in bovine or ovine elongating conceptuses" with no formal proof of a direct role in the induction, maintenance or arrest of the elongation process. In the absence of direct arguments through *in vitro* or *in vivo* knock-down approaches, it thus seems difficult to define the genes which really play the key roles in this complex process.

Recent molecular data based on the analysis of the transcriptome provided also genes or Expressed Sequence Tags (EST), the function of which is not elucidated so far. At first glance, this only lengthened the list of such genes we already had. For example, genes encoding Retinol Binding Proteins (RBP: Trout *et al.* 1991; Dore *et al.* 1994; Yelich *et al.* 1997a), Trophoblast Kunitz Domain Proteins (TKDP: MacLean *et al.* 2003; MacLean *et al.* 2004; Chakrabarty *et al.* 2006) or Pregnancy Associated Glycoproteins (PAG: Green *et al.* 2000; Hughes *et al.* 2003; Klisch *et al.* 2005) were isolated during elongation and further characterised as interesting gene families (TKDP, PAG) or important molecules for the elongation of porcine conceptuses (RBP). However their precise roles remain incompletely understood. Conversely, some of the genes identified through the use of transcriptomic approaches (arrays or SAGE: Serial Analysis of Gene Expression) brought novel perspectives on extra-embryonic development due to the functions they have outside of the elongation process. Among them: (i) an unusual chemokine (CX3CL1; sheep) which could mediate inflammatory reaction or cell adhesion; (ii) a Wnt inhibitor (Dickkopf1; cow) known to be expressed in embryonic tissues during gastrulation or in uterine tissues prior to implantation (Idkowiak *et al.* 2004; Bauersachs *et al.* 2006); and (iii) two developmental factors potentially regulated by estrogens: Stratifin and Midkine (pig; Blomberg *et al.* 2006).

Elongation and embryonic development

Coincident with elongation, growth and differentiation of extra-embryonic tissues, embryonic development proceeds quickly from an inner cell mass, to a germinal disc and ultimately to an embryonic disc which undergoes progressive growth and differentiation (see Fig. 2). These developmental steps have been described extensively over the past decades (Chang 1952; Greenstein *et al.* 1958; Betteridge & Flechon 1988; Vejlsted *et al.* 2006a), but there is little information on the molecular basis of gastrulation in ruminants or ungulates. Molecular markers have been isolated to characterise embryonic poles (anterior versus posterior), embryonic layers (ectoderm, endoderm or mesoderm) and specific structures appearing during gastrulation (e.g., primitive streak), but these markers have mostly been used to identify the stage of development of the embryonic disc. Expression patterns for goosecoid and brachyury were thus reported for ungulates using whole-mount *in situ* hybridisation and used as early markers for the primitive endoderm or the mesoderm, respectively in order to stage the embryos (Meijer *et al.* 2000; van de Pavert *et al.* 2001; Hue *et al.* 2001). Recent studies using either RNA or antibodies as probes revealed additionally interesting specificities in the expression patterns of key gastrulation genes in ungulates as compared to their patterns in the mouse (Guillomot *et al.* 2004; Flechon *et al.* 2004; Degrelle *et al.* 2005; Vejlsted *et al.* 2005; Vejlsted *et al.* 2006b; Blomberg le *et al.* 2006). To our knowledge however, no study has been conducted in ruminants to analyse the interactions between embryonic and extra-embryonic tissues as recently documented in the mouse where reciprocal inductive interactions are involved in (i) defining the antero-posterior pattern of the embryo (Beck *et al.* 2002; Richardson *et al.* 2006) and (ii) preventing precocious differentiation of the extra-embryonic ectoderm (Guzman-Ayala *et al.* 2004).

Therefore, we decided to study these interactions by taking advantage of the ease of non-surgical recovery of bovine conceptuses prior to implantation. We thus selected bovine conceptuses according to their size (ovoid: 1-20 mm, tubular: 50-60 mm, early filamentous: 100-150 mm) and compared the gene expression profiles of each elongation stage (ovoid, tubular and filamentous) while screening a bovine array of about 10 000 cDNA (10K array). A hierarchical clustering based on a set of differentially expressed genes between ovoid, tubular and early filamentous conceptuses revealed two main gene clusters, as well as two main developmental groups (Fig. 3). A major difference appeared between the ovoid versus the tubular and filamentous stages which confirmed on a large repertoire (10K array) that gene expression profiles between tubular and early filamentous conceptuses were not statistically different, as initially observed (1K array; Degrelle *et al.* 2005). In addition to the differences in the size of the conceptus or the extra-embryonic layers formed at those 3 elongation stages, another important feature distinguished them. At both tubular and filamentous stages, the embryonic disc is flat with a clear antero-posterior axis, whereas at the ovoid stage the embryonic disc is a non polarised germinal disc. When related to these morphological observations, the two gene clusters suggest that the transcriptional pattern which distinguishes an ovoid stage from tubular and filamentous stages is, in fact, a pre-gastrulating versus a gastrulating signature. The identity of the genes within these 2 clusters needs now to be explored and understood in the light of embryonic-extra-embryonic interactions. However, the existence of these clusters already extends previous observations where some of the key genes for the embryonic-extra-embryonic interactions in the mouse have been identified in ruminant extra-embryonic tissues (furin or PACE1: Degrelle *et al.* 2005; BMP4 for bone morphogenic protein-4: Cammas *et al.* 2005). Nevertheless, this does not mean necessarily that similar gene networks are involved in establishing early embryonic polarities in ruminant and in rodent extra-embryonic tissues. This topic awaits further studies in ruminants (I Hue, unpublished observations), but reassures us in pro-

posing that the ovoid stage is a critical step in the transition between a spherical and a tubular shape, and also between an ICM and an epiblast developmental stage in bovine conceptuses.

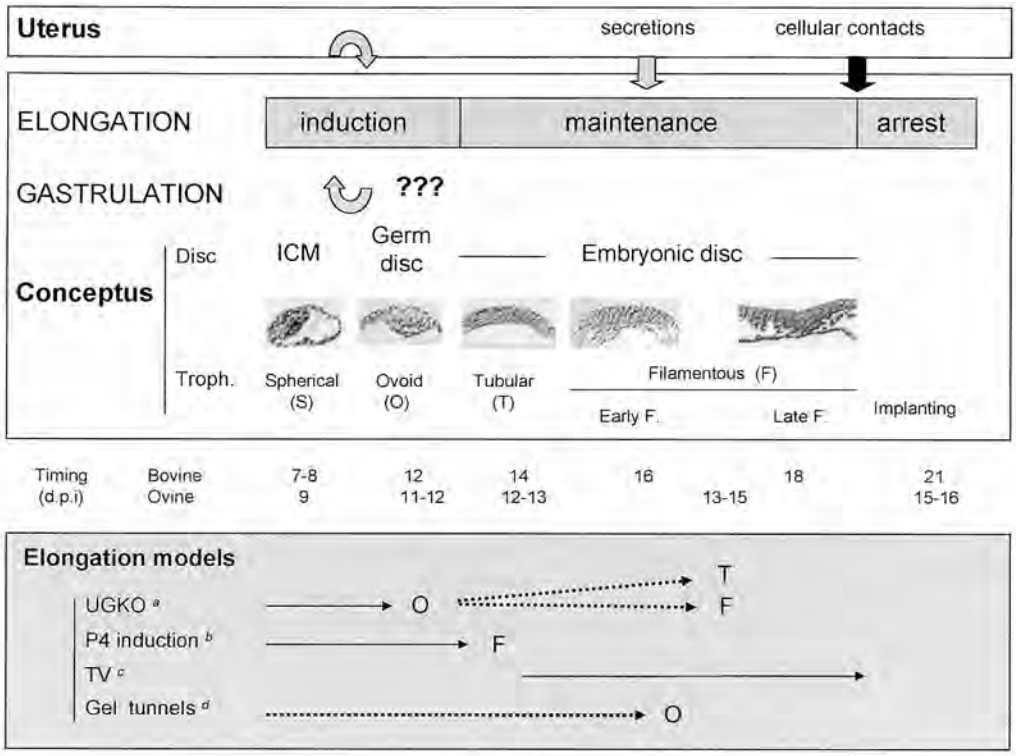

Fig. 2. From a puzzle to an integrated model? In ruminants, the elongation of the conceptus defines the exponential growth of the blastocyst as well as the evolution in its size and shape from a spherical (S) to an ovoid (O), a tubular (T) and finally a filamentous (F) form. These elongating stages also correspond to different stages of embryonic development from an inner cell mass (or ICM) to a germinal disc and finally an embryonic disc. Four main models have been established so far to study what governs the induction and maintenance of the elongation process. Among them: the UGKO model in sheep and the P4 induction model (in sheep and cow) helped analysing the induction phase ( ). The excision of the disc from tubular conceptuses to get trophoblastic vesicles (or T.V., in sheep and cow) demonstrated that uterine secretions maintain the elongation process once properly induced ( ). The culture of spherical blastocysts in gel tunnels confirmed that physical constraints do not fully mimic elongation (in cows). In each model, the elongation stage reached by the blastocyst is given by the appropriate letter: O, T or F. When most of the blastocysts reached the same stage a plain arrow is used but when they reached a stage or another, a dotted arrow is used. However, the events leading to the coordination of elongation and gastrulation have not been cleared so far in ruminants ( ). On the other hand, the loose contacts formed between the conceptus and the uterus clearly mark ( ) the arrest of elongation and the onset of implantation (I). The developmental timing is scaled in days post insemination (or d.p.i) where day 0 marks the onset of oestrus. Italics refer to original reports: (a) Gray *et al.* 2001; 2002, (*b*) Satterfield *et al.* 2006, (c) Heyman *et al.* 1984; Flechon *et al.* 1986, (*d*) Vajta *et al.* 2004; Vejlsted *et al.* 2006a.

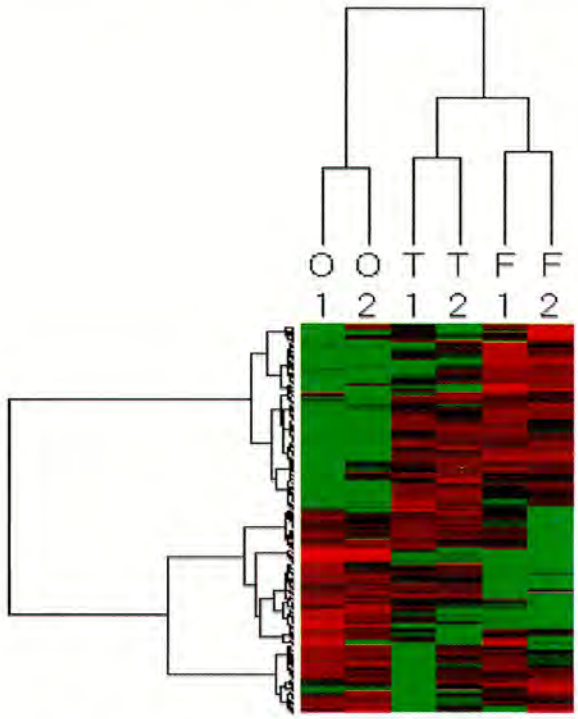

Fig. 3. Pre-gastrulating and gastrulating gene profiles for elongating extra-embryonic tissues. Hierarchical clustering of 239 genes which were differentially expressed between extra-embryonic tissues from ovoid, tubular and early filamentous conceptuses (statistically significant with AnovArray; Hennequet-Antier *et al.* 2005). The elongating stages were defined according to the size of the conceptuses (ovoid (O): 1-20 mm, tubular (T): 50-60 mm, filamentous (F): 100-150 mm) and each probe (O, T, F) corresponded to a pool of four conceptuses. Two independent probes per elongation stage (named: 1, 2) were hybridised to the 10K array. This array will be described in a forthcoming paper (SA Degrelle, unpublished observations). Within the cluster, highly expressed genes are in red and low abundance genes in green. The sizes of the branches within the tree indicate distances between gene sets or elongation stages.

Elongation and uterine environment

Conceptuses of sheep and cattle produce proteins which exert an antiluteolytic effect by inhibiting uterine pulsatile release of PGF2α. As a consequence, continued secretion of progesterone by the corpora lutea stimulates and maintains endometrial functions necessary for growth and elongation of pre-implanting conceptuses (Thatcher *et al.* 1984; Spencer & Bazer 2004). Since the first gene identified in the trophoblast encoded IFN-tau (Farin *et al.* 1990), endometrial genes regulated by IFN-tau have been identified in sheep and cattle together with genes induced by other secretory products of the conceptus in pregnant ruminants (Song *et al.* 2005; Gray *et al.* 2006; Klein *et al.* 2006). Despite the pivotal role of IFN-tau in the embryo-maternal dialog (Thatcher *et al.* 1989; Bazer & Spencer 2006), other cytokines, hormones, growth factors and growth factor receptors or prostaglandins and prostaglandin receptors (Kliem *et al.* 1998; Burghardt *et al.* 2002; Wolf *et al.* 2003; Ashworth *et al.* 2006; Cammas *et al.* 2006), exert

important functions. However, to go one step further in the understanding of the complex signalling pathways between the conceptus and the uterus, one now needs to analyse for temporal and spatial (cell specific) gene expression profiles both before and after elongation of the conceptus.

Reciprocally, blastocyst growth and elongation depend on uterine secretions as evidenced by increased embryonic losses in documented cases of conceptus-uterus asynchrony (Pope 1988; Barnes 2000). This has been confirmed and extended using two experimental models (Fig. 2); one that precludes uterine gland secretions, i.e, the Uterine Gland Knock Out (UGKO) ewe model (Gray *et al.* 2001; Gray *et al.* 2002) and the other that advances uterine secretory activity, i.e, the early progesterone-induced ewe (Satterfield *et al.* 2006) and cow (Garrett et al. 1988) models. Interestingly, 14 days after mating, UGKO ewes presented no conceptus (4 of 8 ewes), growth-retarded conceptuses (3 of 8 ewes) or filamentous conceptuses (1 of 8 ewes), versus 4/4 ewes with filamentous conceptuses in untreated control ewes (Fig. 2). These results demonstrated a direct consequence of lack of uterine glands on conceptus development, that is, no uterine glands and no conceptuses, moderate gland density and retarded to normal conceptuses. On the contrary, exogenous progesterone administration 1.5 days after mating led to increased blastocyst diameters on day 9 and advanced the timing of elongation (filamentous stages on Day 12 already). Unfortunately, not much is known about conceptus development in the ewe model, but secretion of IFN-tau was advanced in cows. There remain many questions, e.g., how does a growth-retarded sheep conceptus (developed in UGKO ewes) appear morphologically when no INF-tau is present in the corresponding uterine flushes? Conversely, in the P4-induced model is the advanced elongation process perfectly similar to that for normal conceptuses? Undoubtedly, gene expression profiling studies on embryonic and extra-embryonic tissues in such situations will provide essential data on the genes or developmental cascades involved in successful uterus/conceptus cross-talk.

In vitro elongation

Mimicking the elongation process *in vitro* has long been a challenge, but has provided many interesting features and questions (Fig. 2). The first experimental approach was to collect *in vivo* elongated conceptuses at the tubular stage (sheep: Day 12; cow: Day 14) cut them into pieces on both sides of the embryonic disc, cultivate them *in vitro* or transfer them back *in utero*. In both species, it appeared that the resulting "trophoblastic vesicles" survived, but did not elongate further *in vitro* whereas they could elongate and produce IFN-tau during 5 day period following transfer back into a synchronous uterine environment (Heyman *et al.* 1984; Flechon *et al.* 1986). As such, it seems that the elongation process and IFN-tau secretion require the uterine environment, but not the embryonic disc (Fig. 2). However, in the absence of any additional results, one does not know whether such trophoblast elongation resembles a normal transition of tubular to filamentous conceptuses.

The second approach to initiating the elongation process *in vitro* was to incubate spherical blastocysts in gel tunnels containing glucose-rich medium (Vajta *et al.* 2004). Compared to cultures in Petri dishes where blastocysts only formed spheres of increasing diameters, cultures in gel tunnels induced a constrained elongation in most blastocysts although very few survived until Day 16 (2/67). The size of the longest blastocyst obtained was about 12 mm which is the size of an ovoid blastocyst developed *in vivo* (1-20 mm; Day 12). Nevertheless, in the few healthy-looking blastocyts (2/67), the inner cell mass had developed, a second cell layer had formed and completed development (Brandao *et al.* 2004), but there was no regression of the Rauber's layer and no formation of a true embryonic disc (Vejlsted *et al.* 2006a). Moreover,

many blastocysts degenerated during culture, even those which grew in Petri dishes, suggesting that the medium used may not have met the metabolic needs of these blastocysts. In light of recent results concerning differential and constitutive gene expression during elongation of ruminant conceptuses, optimising a medium that can (i) sustain protein, nucleic acid, carbohydrate and lipid metabolism of the embryos and (ii) accommodate associated catabolism, seems challenging. Even more challenging are efforts *in vitro* to mimic the uterine environment which is just being decrypted through proteomic studies (Berendt *et al.* 2005).

Elongation: hypotheses and open questions

Based on the models and results presented in this review, three basic features can describe what we know about elongation: (i) it depends on uterine secretions; (ii) it is tightly coordinated with growth and differentiation of the embryonic disc; and (iii) it is not likely to be solely dependent on a physical constraint such as the shape of the uterus. This knowledge is, however, difficult to integrate in a simple model of elongation induction, maintenance and arrest. This is partly due, in our opinion, to: (i) confusion around the start point of elongation and lack of understanding of the mechanisms which induce it; (ii) absence of biological parameters to define the phases of induction or maintenance of conceptus development; and (iii) partial datasets available *in vivo, in vitro* and in experimental models since the uterus or the conceptus, but not both, were usually studied. To gain insights into this simple view of induction, maintenance and arrest of elongation, we will summarize what is known about each phase of the conceptus development.

Induction: After mating, uteri of UGKO ewes maintained conceptuses until Day 9 (spherical stage, 900μm), the conceptuses could not elongate in UGKO ewes, but could in normal ewes. Also, conceptuses from normal ewes did not elongate in uteri of UGKO ewes. Therefore, the mechanism for induction of conceptus elongation is not inherent within the conceptus. However, between the onset of elongation and the parameters that define the ovoid stage: (i) a shape is defined by conceptus length that is about 1.3 times its width (Grealy *et al.* 1996); (ii) a specific programming of the ruminant trophoblast results in its expression of epiblast genes (Degrelle *et al.* 2005); (iii) an intense proliferation of the mural lineage occurs; and (iv) a gene expression profile for extra-embryonic tissues which define pre-gastrulating features. The question is what do the uterine secretions induce? Do they interfere with or influence embryonic-extra-embryonic interactions in any way?

Maintenance: Similar to the situation of normal sheep blastocysts in UGKO ewes, trophoblastic vesicles derived at tubular stages do not elongate *in vitro,* but do so *in vivo*, indicating that induction of conceptus elongation depends on uterine signalling. However, we do not know which components of uterine secretions induce the elongation process or which components of these secretions maintain the conceptus. Do they maintain (i) an elongated shape which does not result solely from physical constraints imposed by the uterine wall since equine conceptuses (though confined as well in long uterine horn) remain spherical from Day 6 to Day 22 (reviewed in Allen 2001) (ii) an ongoing proliferation process (iii) ongoing IFN-tau secretion and/or (iv) a gastrulating signature in tubular and filamentous extra-embryonic tissues?

Arrest: We have known for decades that elongation stops at apposition, the onset of the implanting process (Guillomot 1995; Spencer *et al.* 2004). However, embryonic development does not stop concomitantly, since neural folds and somites appear at that time as if this phase of elongation arrest initiates another stage to achieve synchrony between pre-placental and fetal tissues. These events, however, are outside the focus of this review.

Defining similarities and differences between elongating processes in conceptuses from sheep, cow and pig could help us understand the basic core mechanisms responsible for elongation of conceptuses. Unfortunately, this has not been studied so far although a number of reports on molecular data are available for pigs (Yelich *et al.* 1997b; Wilson *et al.* 2000; Ross *et al.* 2003; Blomberg *et al.* 2005; Lee *et al.* 2005). At present, we have some pieces of the elongation puzzle, but must continue searching for the missing cues until an integrated model can be developed and validated.

Conclusions

Although known for decades, mechanisms responsible for elongation of conceptuses are far from being understood. This is likely due to: i) the numerous interactions with the uterus and the embryonic disc so that only well synchronised processes give rise to a successful pregnancy; ii) the complexity of the process: early processes and late processes which might be distinct but interdependent on each other; iii) cross-talk between cell layers which compose the extra-embryonic tissues; and iv) the inability, to date, to mimic or dissect this complex process *in vitro*.

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