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Molecular evidence for a critical period in mural trophoblast development in bovine blastocysts

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Abstract

Embryonic and extra-embryonic lineages are separated at the blastocyst stage in the mouse at the onset of implantation but well ahead of implantation in most mammals. To provide information on the development of the trophoblast lineage in late-implanting bovine embryos, we combined the use of molecular markers defining embryonic and extra-embryonic lineages in the mouse with a transcriptomic approach dedicated to the early steps of the elongation process, a characteristic feature of blastocyst development in ruminants. In this study, we present molecular evidence for differences between the cow and the mouse in the programming of trophoblast differentiation. This different programming encompasses: (i) the expression of epiblast specifying genes (Oct-4, Nanog) in bovine trophoblast cells at the onset of elongation, (ii) the transcription of proliferation markers in early elongating blastocysts, (iii) the early detection of trophoblast-specific transcripts related to extra-embryonic tissue's differentiation (Hand1, Ets2, IFN-tau) and (iv) the identification of a new transcript (c12) which displays a reciprocal pattern to that of Oct-4 and Nanog genes in the embryonic cells and for which no equivalent has thus far been found in the mouse. Altogether, these results tended to show that early elongation is a critical transition in bovine trophoblast development.
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Introduction

In mouse embryos, the first cell fate decisions occur before implantation and separate embryonic from extra-embryonic lineages, giving rise to the foetus and the foetal part of the placenta. These decisions become visible at the blastocyst stage (3.5 days post-coitum or dpc), when the inner cell mass (ICM) and the trophectoderm are easily recognized, even if the first diverging precursors appear at the morula stage (for a recent review, see Rossant, 2004). Two trophectoderm cell types can then be distinguished: the cells which cover the ICM, or polar trophoblast, and those which surround the blastocoelic cavity, or mural trophoblast. The polar trophoblast proliferates actively and the signals which stimulate this proliferation originate from the ICM and act only locally. Polar trophoblast cells also give rise to the extra-embryonic ectoderm (ExE) and differentiate into the ectoplacental cone (EPC) at 5.5 dpc. As for mural trophoblast cells, they surround the blastocoelic cavity, are not in close contact with the ICM, and do not benefit from the trophoblast growth factors emitted by the embryonic cells. They exit the mitotic cell cycle, enlarge and eventually go through rounds of DNA replication without mitosis to become polyploid and form primary giant cells at 5.5 dpc (Cross, 2005). Thus, being in the vicinity of embryonic cells ensures restricted proliferation of mouse trophoblast cells to the polar lineage.

What differentiates polar and mural cells within the trophoblast lineage remains largely unknown. Up to now, the

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development of the trophoblast lineage was considered as a default pathway resulting from the down-regulation of the Oct-4 transcription factor, one of the genes involved in the ICM/epiblast lineage (Nichols et al., 1998; Niwa et al., 2000; Pesce and Scholer, 2001). Recent studies, however, have shown that trophoblast specification and maintenance are positively controlled in the mouse blastocyst by specific transcription factors (Donnison et al., 2005; Strumpf et al., 2005). The transcription factor Cdx2 first specifies the trophoblast versus the ICM cell fate, while Eomesodermin (Eomes) participates in the control of polar versus mural trophoblast cell fate and Elf5, a member of the large Ets family, is involved in the control of the ExE versus the EPC decision within the polar lineage. Such data introduces the novel concept of a positive control in the specification and maintenance of the trophoblast lineage. Moreover, since Cdx2 has been shown to restrict the expression of the Oct-4 gene to the ICM (Strumpf et al., 2005), cell fate specification and maintenance at the blastocyst stage results more probably from a coordinated network of positive and negative regulations of gene expression than from sequential on/off molecular events (Rossant, 2004). If so, it can be considered that Oct-4 and Nanog promote in the ICM an epiblast cell fate while repressing the trophoblast-specific and endoderm genes, respectively. Once committed, the development of each cell type, ICM and trophoblast, requires cell fate maintenance and lineage-specific differentiation.

In most mammals, although not in rodents or higher primates, the polar trophoblast, also called “Rauber’s layer”, quickly disappears after blastocyst expansion and hatching (Viebahn, 1999) and there is thus no anatomical equivalent to the mouse ExE or EPC. Subsequently, the trophoblast grows and differentiates from the cells which surround the blastocoelic cavity but not from the ones which cover the ICM. In ruminants, the trophoblast elongates exponentially and reaches more than 150 mm before implantation. Several observations provide clear evidence that intense trophoblastic cell multiplication precedes cellular differentiation (Chang, 1952; Wintenberger-Torres and Flechon, 1974) and that early elongation relies on cell growth and cell shape remodeling (Betteridge and Flechon, 1988). Nevertheless, no molecular data has been put forward to characterize the intense multiplication or proliferation of the trophoblastic cells at the onset of the elongation phase. The genes identified so far during elongation are related to molecules involved in foeto-maternal exchanges before implantation and to functions underlying such a differentiation. It has thus been learned (i) that molecules such as interferons (IFN-tau; Farin et al., 1990), prostaglandin synthases (PGHS-2; Charpigny et al., 1997), protease inhibitors (TKDP family; MacLean et al., 2003) and pregnancy-associated proteins (PAG family; Green et al., 2000) are increasingly transcribed or secreted when the bovine (or ovine) trophoblast elongates and (ii) that general metabolism, protein trafficking and cell proliferation are characteristic of the onset of elongation whereas cellular interactions, cell to cell signaling and cell adhesion become predominant at the end of elongation (sheep: Cammas et al., 2005; cattle: Ushizawa et al., 2004). However, questions related to the development of the trophoblast or the epiblast lineage remained elusive in ruminants until van Eijk et al. (1999) reported the expression of Oct-4 in the trophoblast of bovine blastocysts. Since then, the detection of Oct-4 mRNA or protein in the trophoblast of early expanded blastocysts from different mammalian species has been extensively investigated (Hansis et al., 2000; Kirchhof et al., 2000; Mitalipov et al., 2003; Kurosaka et al., 2004; He et al., 2005; Vejlsted et al., 2005), but the expression of transcription factors which positively specify the trophoblast in the mouse, such as Cdx2, remain poorly documented (Ponsuksili et al., 2001; Cammas et al., 2005; Hall et al., 2005).

In the bovine species, blastocysts are formed several days after insemination (7 dpi) but their apposition to the uterus, the first sign of implantation, takes place about two weeks later (21 dpi; Guillomot, 1995). By that time, bovine blastocysts have already initiated the gastrulation process and the trophoblast has elongated dramatically from 150 μm to 300 mm (Chang, 1952; Greenstein et al., 1958; Betteridge and Flechon, 1988; Hue et al., 2001; Maddox-Hyttel et al., 2003). This large developmental window between differentiation of the first embryonic lineages and the onset of implantation makes late implanting mammals such as the bovine species a particularly attractive model in uncoupling the mechanisms of trophoblast growth and differentiation from those of implantation. By combining these developmental advantages of cattle blastocysts with the knowledge gathered on the mouse model in terms of molecular markers of trophoblast and epiblast lineages, we define here a period in the development of the mural trophoblast when cells are still transcribing epiblast genes while already transcribing several trophoblast-specific genes. Such data could be used for a functional definition of the ovoid stage during the development of the blastocyst in late implanting mammals.

Materials and methods

Bovine blastocysts

Estrus synchronized heifers of the Charolais breed were inseminated (day 0) and day 12 to day 17 blastocysts were collected by non-surgical flushing in warm PBS. Ovoid blastocysts (1–12 mm) came from collects at 12 dpi (day post-insemination) whereas tubular and early filamentous stages (50–60 mm and 140–160 mm) were obtained at 14 to 15 and 16 to 17 dpi, respectively. For economy and ease, spherical stages were produced after in vitro maturation, fertilization and development (Menck et al., 1997). The immature oocytes were punctured from cow ovaries of different breeds, originating from a local slaughterhouse, but the same semen was used for in vivo and in vitro insemination. The quality of such in vitro produced blastocysts was evaluated at the end of the culture (7 dpi; Shehu et al., 1996) and their viability confirmed by the birth of healthy calves after transfer into recipient heifers (Heyman et al., 2002).

In situ hybridization

For whole-mount in situ hybridization, whole spherical (n = 43) and ovoid (n = 10) blastocysts or isolated embryonic discs dissected out from tubular (n = 5) and early filamentous (n = 5) blastocysts were fixed (4% paraformaldehyde), stored and hybridized with a DIG-labeled riboprobe as described in Hue et al. (2001). The hybridized blastocysts or embryonic discs

were then mounted in mowiol, photographed, embedded in Technovit 8100 (Heraeus Kulzer) and sectioned (6 μm). The sections were photographed with a Coolsnap camera (Photometrics). For in situ hybridization on tissue sections, whole ovoid (n = 14) and filamentous (n = 5) blastocysts were fixed (4% paraformaldehyde), stored, sectioned (10 μm frozen sections) and hybridized as described in Cammas et al. (2005) with DIG-labeled riboprobes (RNA DIG-labeling kit, Roche Diagnostic). Frozen sections (10 μm) from bovine placentas collected at 69 days of pregnancy were a kind gift from Dr M. Guillomot (BDR, INRA, France). The hybridized sections were observed and photographed using a microscope, a digital camera and software from Olympus. The bovine cDNA fragments encoding Oct-4, Nanog A, Nanog B, Sox-2, Gata-6 or Hand1 were derived from PCR amplifications, sequenced, cloned into pGEMT-Easy (Promega) and in vitro transcribed using the Sp6 RNA polymerase (anti-sense probes). The other cDNA fragments (Furin, c12 and c93) were isolated from the cDNA library established at the ovoid stage, re-cloned into pGEMT-Easy and in vitro transcribed using the Sp6 RNA polymerase except for c93 (T7 RNA polymerase to generate the anti-sense probe).

RT-PCR

Total RNA was extracted from the embryonic and extra-embryonic tissues of spherical (n = 23), ovoid (n = 5) and early filamentous (n = 3) blastocysts with RNeasy Mini Kit according to manufacturer's instructions (Qiagen). The reverse transcriptions were done on 1 μg of early filamentous RNA and on the whole RNA extraction for the other stages, using 200 U of Superscript II (Invitrogen) and 500 ng of oligo(dT). The 1st cDNA strand was diluted 1:5 and submitted to PCR amplification in 50 μl of a 1× PCR buffer: 0.4 mM dNTP, 0.2 μM of specific primer (MWG) and 1 U of Taq polymerase (QBioGene). 0.5, 1 or 2 μl of each RT was used in the PCR reactions: 0.5 μl for Oct-4, Sox2, Ets2, IFN-tau and β-actin; 1 μl for Gata-6, Cdx2, Elf5 and Hand1 and 2 μl for Fgf4, Opn, Nanog, and Eomes. β-actin, Ets2, IFN-tau, Oct-4 and Sox2 were submitted to 35 PCR cycles, Cdx2, Elf5, Eomes, Fgf4, Gata-6, Hand1, Nanog and Opn to 40 cycles. See annealing temperatures in Table 1 and contact us for details on the primers.

RNA amplification and aRNA labeling

Total RNA from ovoid (n = 30), tubular (n = 9) and early filamentous (n = 5) extra-embryonic tissues was extracted with RNA-Plus™ (QBioGene). Amplified RNA from each sample was then synthesized starting from 1 μg of RNA with the MessageAmp™ aRNA Kit (Ambion) according to the manufacturer's instructions. aRNA purification was performed on Mini Quick Spin RNA columns (Roche Diagnostic) and 500 ng aRNA were labeled with [α-^{33}P]dATP (Decraene et al., 1999).

Table 1
PCR conditions and Amplicon identifications

Transcript name	T °C annealing	Product length (bp)	Accession number GenBank
Oct4	58	337	DQ126156
Sox2	58	514	DQ126150
Fgf4[a]	55	196	U15969
Opn	55	783	DQ126149
Nanog	58	357	A: DQ126154
		325	B: DQ126153
Gata6	58	541	DQ126151
Cdx2	55	140	DQ126146
Eomes	55	526	DQ126147
Elf5	56	644	DQ126155
Hand1	55	195	DQ126152
Ets2	58	227	DQ126148
IFN tau[b]	56	620	NM_001015511
βactin	60	319	AY141970

[a] Daniels et al., 2000.
[b] Kubisch et al., 2003.

Northern blot

Total RNA from somatic tissues was extracted with RNA-Plus™ (QBioGene) and total RNA from ovoid, tubular and filamentous extra-embryonic tissues came from the same extractions as for RNA amplifications. Northern blotting was performed with the NorthernMax™ kit (Ambion) according to manufacturer's instructions. 100 ng to 500 ng of RNA was loaded onto a 1% agarose gel, transferred to a BrightStar-Plus™ membrane (Ambion) and hybridized to ^{32}P-labelled anti-sense riboprobes (see aRNA labeling) at 68°C. The membranes were exposed to phosphor-imaging for 7 days. Phosphor screens were scanned using a STORM 760 (Molecular Dynamics).

cDNA library construction

The ovoid blastocysts used to construct the library were collected in Belclare (TEAGASC, Ireland) by Dr J.M. Sreenan. Total RNA was extracted with RNA-Plus™ (QBioGene) and 1.6 μg of RNA was used for double stranded cDNA synthesis using the Cap Finder cDNA kit from Clontech (Ozyme). The first cDNA strand generated by reverse transcription was amplified for 19 PCR cycles (95°C 15 s, 68°C 5 min) to generate the second strand and cloned in the pCR2.1 vector (TA cloning kit; Invitrogen) with a 1.5:1 ligation ratio. Transformation in TOP10F' yielded about 1.7 ± 10^6 colonies per μg of double stranded cDNA. Among the 4 ± 10^4 colonies plated, 97% were recombinant and insert sizes ranged from 0.5 to 3 kb.

Macro-array construction

Bacterial clones were randomly picked from the plated library, arrayed in 96-well plates (ATGC) and cultured in LB medium supplemented with ampicillin (50 μg/ml). cDNA inserts were amplified by PCR using flanking primers (PCRp1, see Cap Finder cDNA kit, Clontech). Each reaction contained 2 μl of bacterial culture, 1× PCR buffer and 1 U of Taq polymerase (QBioGene). The amplified products were run on a 2.5% agarose gel and selected for further use. After spotting, the DNA was denatured (NaOH 0.5 M, NaCl 1.5 M; 5 min), neutralized (Tris 0.5 M, NaCl 1.5 M; 5 min) and fixed to the membrane (2 h, 80°C). 1850 inserts were spotted onto nylon N+ membranes (8 cm × 12 cm, Amersham Biosciences) with a 5 × 5 pattern (QPixII). Internal controls within the array corresponded to 70 spots. The "bcai" library is indexed as "#FJB" (http://www.tigr.org) or "15979" (http://www.ncbi.nlm.nih.gov) and the bacterial clones are available under request at the CRB GADIE (Francois.Piumi@jouy.inra.fr).

Expressed sequence tag sequencing

1850 EST were sequenced one way with M13 reverse primer (MWG). Quality treatment processing was carried out according to a classical procedure: vector and adapter masking, insert detection and calculation of sequence validity using criteria such as sequence length and Phred score. As a result, 1097 bcai sequences were considered valid and submitted to the EBI database using INRA-AGENAE bio-informatics facilities (http://sigenae.org). The nucleic acid comparisons between these sequences and the Bos taurus Gene Index (BtGI, Cattle Release 10 as of 13 May 2004, http://www.tigr.org) used stringent BLASTN criteria so that selected hits were defined as >90% nucleic identity over more than 90% of the shortest sequence. TIGR gene indices provided links from TC (Tentative Consensus sequences) to Gene Ontology terms.

Macro-array hybridization, image acquisition and quantification

Each aRNA probe was hybridized to 4 macro-array replicates using ExpressHyb™ Hybridization Solution (Clontech) at 68°C overnight. Membranes were washed four times in 2× SSC, 1% SDS and once in 0.1× SSC, 0.5% SDS at 68°C for 30 min each. They were then exposed to phosphor-screens for 7 days. The hybridization signals were quantified with the Imagene 3.1 software from BioDiscovery (Proteigene) on the PICT platform (INRA, Jouy en Josas). The positive and negative controls were as expected in all the hybridizations.

Gene expression data analysis

The experimental design leading to the macro-array data was based on the hybridization of 3 embryonic stages. For each stage, two independent aRNA probes were generated. Each one was hybridized to 4 membranes and each membrane contained 1097 valid sequences. Mitochondrial sequences (n = 110) were left aside so that 23,688 pieces of data (3*2*4*987) were Log2-transformed and standardized within each stage (for each spot, averaged intensity of the two independent aRNA probes). Differential analysis was performed with AnovArray (http://www-mig.jouy.inra.fr:stat/AnovArray; Hennequet-Antier et al., 2005), using the following linear model: $y_{ijk} = \mu + \alpha_1 + \beta_j + \gamma_{ij} + \xi_{ijk}$, where y is the Log2-transformed and standardized intensity for each spot, μ is the overall mean, α_i is the effect of the developmental stage i (i = ovoid, tubular or filamentous), β_j is the effect of the gene j (j = 1,..., 987 and here gene means EST), γ_{ij} is the effect of the interaction between developmental stage and gene. The residuals ξ_{ijk} (k = 1 to 8 and k means replicate) are assumed following a Gaussian distribution (mean 0 and variance σ^2). The null hypothesis, no interaction between gene and stage, was tested. To determine which genes were differentially expressed between the 3 developmental stages, we used the macro called "differential analysis" considering a homogeneous variance for all the genes (HOM option) since this was reasonable for this dataset. Using a multiple comparison test FDR (False Discovery Rate) at the threshold 5%, 8 transcripts were identified as differentially expressed between ovoid, tubular and filamentous stages.

Institutional agreements

In vitro-developed embryos (7 dpi) were produced by the certified team of INRA, accredited by the ministry of agriculture and the veterinary services No. FRPB780 and FRTB910. To recover late in vivo-developed embryos, uterus samples (day 24 and term) or adult somatic tissues, animals were humanly put down in the accredited experimental slaughterhouse of INRA under the supervision of veterinary services.

Results

Gene expression profiles of bovine epiblast and trophoblast lineages

In the present work, elongating bovine blastocysts were collected between 12 and 17 days post-insemination (dpi) and identified as ovoid, tubular or early filamentous depending on their shape and size (Figs. 1B–D). Concomitantly to the growth of the extra-embryonic tissue, the embryonic part evolves from an inner cell mass to a germ disc and a flat embryonic disc (Figs. 1E, H) and both differentiate during these days as described (Chang, 1952; Betteridge and Flechon, 1988; Maddox-Hyttel et al., 2003). Bovine spherical blastocysts were

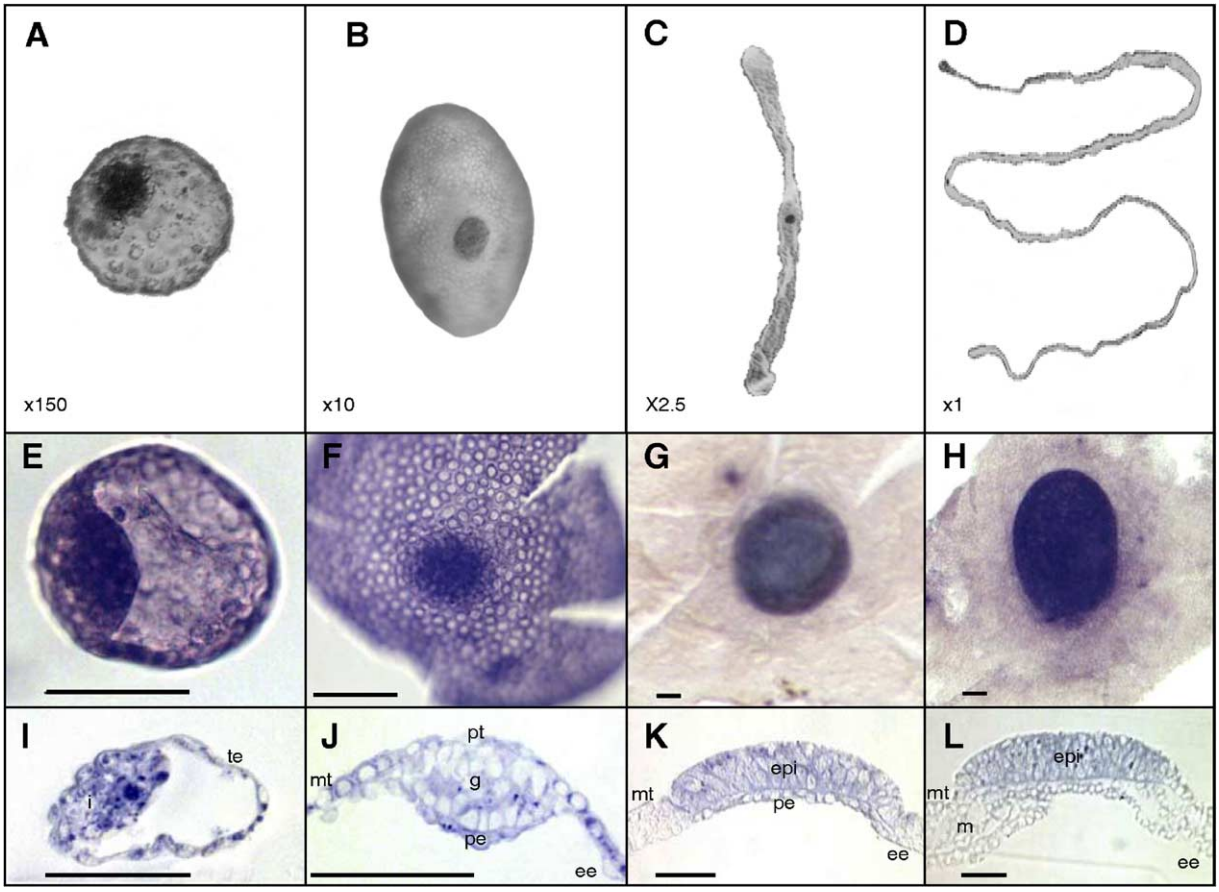

Fig. 1. Growth and differentiation of embryonic and extra-embryonic tissues in bovine pre-implanting blastocysts. Spherical (A), ovoid (B), tubular (C) and early filamentous blastocysts (D) increased in size from 150 μm to 130–150 mm. Whole-mount in situ hybridization with a DIG-labeled Oct-4 anti-sense probe on spherical and ovoid blastocysts (E–F) together with isolated embryonic discs from tubular and early filamentous blastocysts (G–H). Lateral (E) or dorsal (F–H) views of one representative hybridization per stage. Transverse sections of the Oct-4 hybridized blastocysts (I) or embryonic discs (J–L) confirmed the cellular location of the transcripts. Scale bar: 100 μm. Oct-4 sense probe did not give any signal. i: inner cell mass, te: trophoectoderm, pt: polar trophoblast, mt: mural trophoblast, g: germ disc, pe: primitive endoderm, ee: extra-embryonic endoderm, epi: epiblast, m: mesoderm.

in vitro produced but displayed the expected morphology with both trophoblast and inner cell mass lineages established at 7 dpi (Fig. 1). To work out the hypothesis of a delayed schedule in the restricted expression of the genes which specify each lineage due to the late implantation time, we gathered molecular arguments on pre-implanting bovine blastocysts using the expression of genes known to be essential to the epiblast, trophoblast and endoderm lineages in the mouse.

We first analyzed three positive regulators of the epiblast lineage: Nanog and Oct-4, along with Sox2 which is considered to share a role with Oct-4 in maintaining the ICM cell fate (Avilion et al., 2003). Whole-mount in situ hybridization performed with a bovine Oct-4 probe revealed Oct-4 transcripts in the ICM/epiblast lineage at each of the blastocyst stage (Figs. 1E–L). The Oct-4 mRNA was also visible in trophoblast cells of most spherical and ovoid blastocysts (Figs. 1I–J) since 40 out of the 43 examined were strongly positive at the spherical stage and 9 out of 10 were positive at the ovoid stage. Oct-4-specific amplifications confirmed those results both for spherical and ovoid extra-embryonic tissues (Figs. 2B1–B2). Oct-4 transcripts were also clearly identified in the germ disc and the epiblast of both ovoid and filamentous stages after in situ hybridization on blastocyst sections (Fig. 3). Though nicely expressed in the extra-embryonic mesoderm of filamentous blastocysts, these transcripts were hardly visible in trophoblast cells at the ovoid stage. Similarly, Sox2 transcripts were easily detected in embryonic tissues by PCR (Fig. 2) and in situ hybridization (Fig. 3), namely: in the germ disc and the epiblast of elongating blastocysts. They were also faintly expressed in the extra-embryonic tissues at spherical and ovoid stages (Fig. 2) but hardly detectable in trophoblast cells of ovoid blastocysts (Fig. 3A). In addition, Nanog amplifications gave a clear signal in all embryonic and extra-embryonic tissues except at the spherical stage (Fig. 2B1). Behind the unique band seen on the gel after amplification (360 bp), two Nanog variants were distinguished by sequencing: one with 71% (Nanog A) and the other with 99% (Nanog B) identity to the predicted bovine NANOG protein (XP_583863). Interestingly, Nanog A showed 90% identity to the human NANOG protein (305 amino acids) and both variants showed an open reading frame of 118 (Nanog A) or 107 (Nanog B) amino acids. The two Nanog variants were expressed in the germ disc and the epiblast of ovoid and early filamentous blastocysts, with a stronger labeling for Nanog B than Nanog A. Compared to Sox2, Nanog B transcripts were not restricted strictly to the germ disc but appeared in the cells which covered the germ disc (polar trophoblast; Fig. 3A). Thus, the co-expression of Oct-4, Sox2 and Nanog transcripts in the trophoblast of ovoid blastocysts suggested by the PCR data, was confirmed for Oct-4 and Nanog (Figs. 1J and 3A, respectively).

These results were in agreement with the hypothesis of a delayed schedule in the restriction of epiblast-specific genes to primitive ectoderm cells in bovine embryos. To further confirm these observations, we also characterized the expression pattern of two downstream effectors of the above transcription factors: (i) the Fgf4 gene which is activated by both Oct-4 and Sox2 in the ectodermal lineage and (ii) the Osteopontin gene (Opn or Spp1) which is activated by Oct-4 and another co-factor in the endoderm lineage of the mouse embryo (Yuan et al., 1995; Ambrosetti et al., 2000; Guo et al., 2002). We found Fgf4 and Opn bovine transcripts expressed in the embryonic tissues of each blastocyst stage (Fig. 2), Fgf4 mRNA in mural trophoblast cells of spherical blastocysts and Opn mRNA in the extra-embryonic tissues of ovoid and filamentous blastocysts.

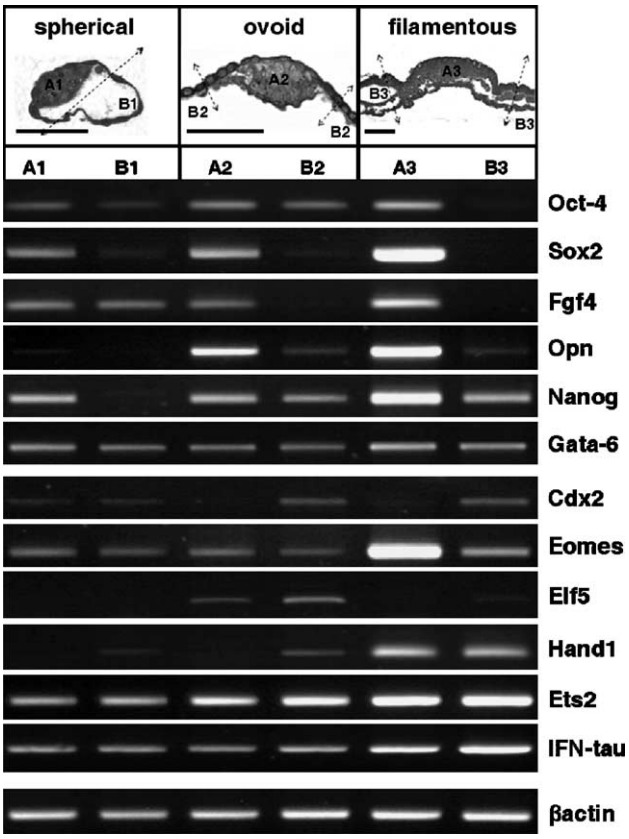

Fig. 2. Expression in bovine pre-implanting blastocysts of epiblast, trophoblast and endoderm genes. Qualitative RT-PCR was performed on embryonic and extra-embryonic tissues of spherical (1), ovoid (2) and early filamentous (3) blastocysts. The embryonic (A) and extra-embryonic (B) tissues were initially sectioned as illustrated by the dotted arrows on the top of the figure. The expression profile of 4 genes from the ICM/epiblast lineage (Oct-4, Sox2, Fgf4, Nanog), 5 from the trophoblast lineage (Cdx2, Elf5, Hand1, Ets2, IFN-tau), 2 from the endoderm lineage (Gata-6, Opn) and one important in both trophoblast and mesoderm formation (Eomes) was established on both tissues (A, B) and all stages (1, 2, 3). Actin β was used here as positive control of the reverse transcriptions. One fifth of each PCR reaction was loaded onto 2 to 3% agarose gels depending on the size of the amplicons. The identity of each amplified product was confirmed by sequencing (Genbank accession numbers in Table 1).

We then asked whether the persistent expression of Oct-4 and Nanog in spherical and ovoid trophoblast cells was not due to a weak expression of negative regulators such as Cdx2. Qualitative RT-PCR indicated that Cdx2 was expressed in the mural trophoblast of spherical and ovoid blastocysts (Fig. 2), but was not detectable in the polar layer at the ovoid stage in the conditions used in situ for the other probes. Eomes, which in the mouse has a dual role in trophoblast but also in mesoderm formation (Russ et al., 2000), was found by PCR in all embryonic and extra-embryonic tissues of bovine elongating blastocysts (Fig. 2). More precisely, Eomes expression

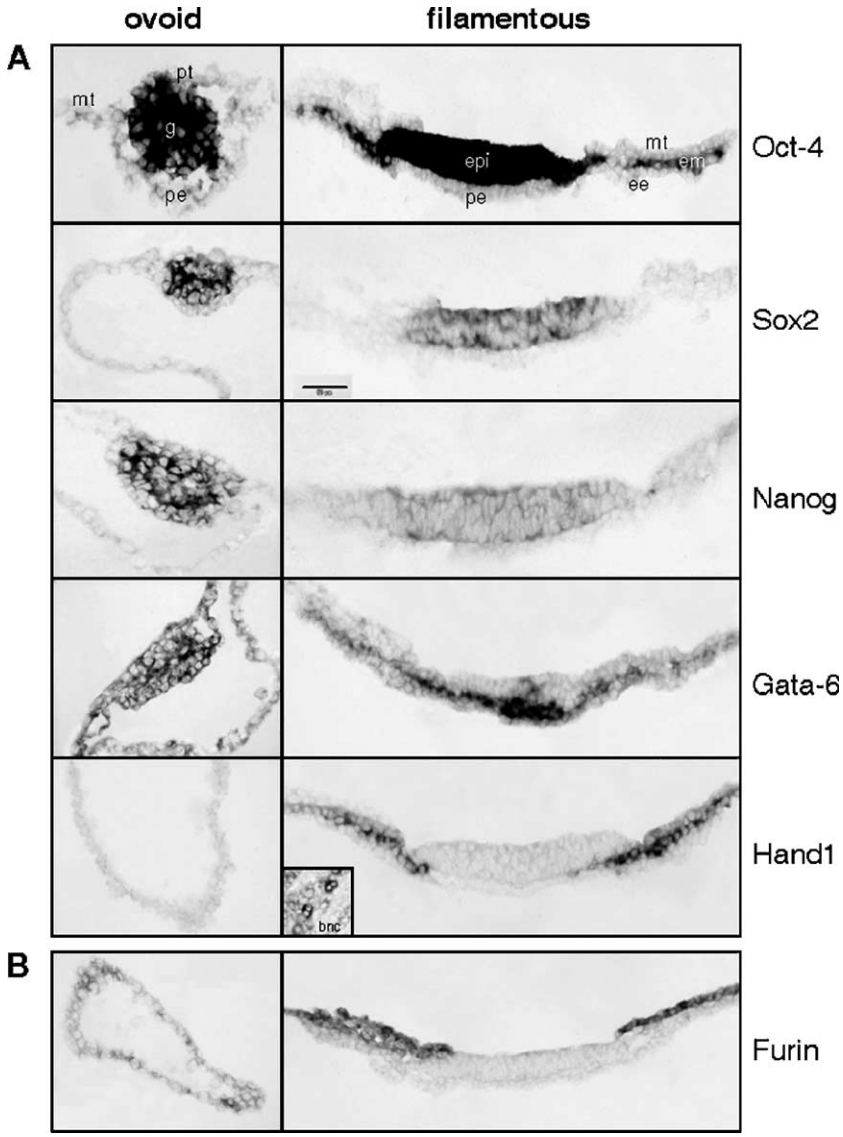

Fig. 3. In situ localization in pre-implanting bovine blastocysts of several genes expressed in embryonic or extra-embryonic tissues. Transverse sections from ovoid and early filamentous blastocysts were hybridized with DIG-labeled RNA probes. In A, bovine probes encoding epiblast (Oct-4, Sox2, Nanog), endoderm (Gata-6) and trophoblast (Hand1) genes in mouse embryos were hybridized to ovoid and filamentous blastocyst sections. The Hand1 probe was also hybridized to day 69 placenta sections (left bottom, Hand1 panel). The bovine variants Nanog A and Nanog B were hybridized on ovoid and filamentous blastocyst sections. A picture of Nanog B is shown at the ovoid stage (A) and a picture from Nanog A is shown at the filamentous stage. In B, a bovine probe isolated from the cDNA library established at the ovoid stage and encoding a PACE1 protease (Furin) was hybridized to ovoid and filamentous blastocyst sections. Sense probes did not give any signal. Each pattern was established on 2 blastocysts per stage and 1 to 3 sections per blastocyst. Scale bar: 50 μm. epi: epiblast, g: germ disc, pe: primitive endoderm; pt: polar trophoblast, mt: mural trophoblast; em: extra-embryonic mesoderm; ee: extra-embryonic endoderm, bnc: bi-nucleated cells.

was located in the epiblast of bovine blastocysts at the filamentous stage but was not found in mural trophoblast cells (data not shown). Elf5, transcription factor sustaining proliferation in the polar trophoblast lineage of mouse embryos (Donnison et al., 2005), was only detected by PCR in bovine blastocysts at the ovoid stage (Fig. 2). While Cdx2 and Eomes were detected at the ovoid stage when both a bigger amount of probe and an extended revelation time were combined (data not shown), Elf5 remained undetectable. As for many genes in the mouse model, this observation could be interpreted as a different detection threshold between RT-PCR and ISH (http://www.informatics.jax.org), or a need for Elf5-specific ISH optimization.

Based on the morphological differences which appear in the extra-embryonic tissues from one blastocyst stage to the next, we further tried to find out when transcription factors involved in trophoblast differentiation and placenta formation could be first expressed. Hand1 mRNA, known as an essential factor in the differentiation of mouse trophoblast giant cells, was detected in the extra-embryonic tissues at each blastocyst stage though mainly expressed at the early filamentous stage (Fig. 2), in the extra-embryonic mesoderm cells which migrate out of the bovine embryonic region. Interestingly, this transcript was still detected at more advanced stages (D69) in the fetal part of placental tissues namely the bi-nucleated cells, the bovine equivalent of

mouse giant cells (Fig. 3). The Ets2 gene, studied in ruminants and known for its role in the mediation of growth factor signaling to key genes of trophoblast development (Roberts et al., 2004), was expressed at each blastocyst stage concomitantly with one of its targets: the interferon-tau gene (Fig. 2) and both were located in the trophoblast (see Ezashi et al., 2001; Farin et al., 1990). In addition, the transcript of GATA-6, a positive regulator of the mouse endoderm lineage, was found in both embryonic and extra-embryonic tissues at each blastocyst stage after specific PCR amplifications (Fig. 2). It was found in embryonic and extra-embryonic mesoderm cells at the early filamentous stage, but also in the germ disc, the primitive endoderm cells and the extra-embryonic tissues at the ovoid stage (Fig. 3).

Altogether, these results suggested that (i) the persistent expression of Oct-4 and Nanog transcripts in trophoblast cells of spherical and ovoid bovine blastocysts is associated with a weak expression of Cdx2, one of their negative regulators, (ii) mural trophoblast cells display a transient "undifferentiated proliferative" phenotype at the ovoid stage when they faintly co-express Oct-4, Nanog, Eomes and Elf5 and (iii) genes involved in trophoblast differentiation processes (Ets2, IFN-tau, Hand1) start to be transcribed as early as the spherical stage.

New expression patterns at the onset of elongation

Based on the above data using mouse developmental markers to show a different programming of trophoblast differentiation in the cow from that of the mouse, we looked for bovine genes to characterize specific processes in the proliferation and/or the early differentiation of the bovine trophoblast. To this aim, we first established a bovine cDNA library at the ovoid stage to focus on early elongation processes. From the 1850 cDNAs isolated, 914 (49%) provided a repertoire of non-overlapping sequences (see details in Math and Meth), half of which matched with already annotated sequences from the Bovine Gene Index. One fourth of them however were linked to a Gene Ontology term (GO). This part of the repertoire included functions related to cell growth and maintenance, cell communication, development and physiological processes (indent 2 of the GO tree; Fig. 4). Among them, some were especially relevant to previous observations reporting cell multiplication and cell shape remodeling at the onset of elongation, namely: cell cycle (40 cDNA), cell proliferation (25 cDNA) or cell organization and biogenesis (50 cDNA). In addition, developmental functions such as gene silencing through chromatin remodeling, cell adhesion, cell migration and epithelial organization could somehow be involved in late specification events or early differentiation pathways. Interestingly enough, one copy of the DAB2 gene (out of 914), one of the effectors of the mouse GATA-6 transcription factor which positively specifies the endoderm lineage (Morrisey et al., 2000; Yang et al., 2002), was identified within the repertoire and located to the ovoid germ disc (data not shown). Similarly, one copy of the Furin gene (also termed PACE1), which produces one of the SPC proteases secreted by the extra-embryonic ectoderm that act on neighboring tissues to specify antero-posterior asymmetry in the mouse (Guzman-Ayala et al., 2004), was also identified in bovine trophoblast cells of ovoid and early filamentous blastocysts (Fig. 3B).

Despite the interesting features of this ovoid repertoire, we did not know how many transcripts from the collection could describe early elongation processes. As a matter of fact, the Furin transcript was found in the ovoid repertoire but was also expressed at a later stage (Fig. 3B). To identify transcripts mainly involved in early elongation processes, we proceeded to a differential screen of the ovoid arrayed repertoire with ovoid, tubular and early filamentous extra-embryonic tissues as probes. Differential expression patterns were statistically investigated, leading to the identification of 8 transcripts dissimilarly expressed among the 3 developmental stages. Five transcripts corresponded to genes already characterized during bovine trophoblast differentiation and encoded an interferon (IFN-tau), two protease inhibitors (TKDP1, TKDP5), a prostaglandin synthase (PGHS-2) and a pregnancy-associated protein (PAG11). The three other cDNA identified here, namely Nap1L1, c12 and c93, were analyzed by Northern blot to determine the stage when they were preferentially or specifically expressed (Fig. 5). It turned out that the Nap1L1 mRNA (Nucleosome-associated-protein 1) was preferentially expressed at the ovoid stage though at a lower level in tubular and filamentous stages, in chorion (24 dpi) and in adult somatic tissues. Nap1L1 has been identified in cleaving bovine embryos before the blastocyst stage (Tesfaye et al., 2003) and is known to be a cell proliferation predictor in human cancers (Line et al., 2002). The other sequences corresponded to Expressed Sequence Tags (EST). Since each of them revealed a major transcript on Northern blots (c12: 1.6 kb; c93: 1.8 kb), we considered them as potential novel genes (Fig. 5). The c12 transcript was partly identical to EST contained in bovine cDNA libraries established on spherical blastocysts (8 dpi; CV978897, DN8843633). In our study, c12 mRNA was mainly detected at the ovoid stage in polar and mural trophoblast cells but also in primitive endoderm cells (Fig. 6A). It was no longer detectable in extra-embryonic endoderm cells at the tubular stage but present in mural trophoblast cells with a discontinuous pattern (Fig. 6B). Interestingly enough, its pattern of expression was strictly nuclear and reciprocal to the one visualized for Oct-4, Nanog and Sox2 in the germ disc (Fig. 3A). Conversely, the c93 cDNA identified in bovine utero-placental libraries as BP112447 or CR851954 was exclusively (Fig. 6) and increasingly (Fig. 5) expressed in the trophoblast during elongation. It was also expressed in the fetal part of the placenta as late as term, in the uterus and weakly in small intestine, lung and mammary gland (Fig. 5). Whether this gene has a similar function in these tissues is unknown, but one of the common features one can first think of is that they all contain epithelial cells. Although not much can be inferred from this study regarding c12 and c93 functions, these transcripts do have an interesting expression pattern but without any mouse or human orthologue identified so far and do not give any hybridization signal on 6.5 to 7.5 dpc mouse

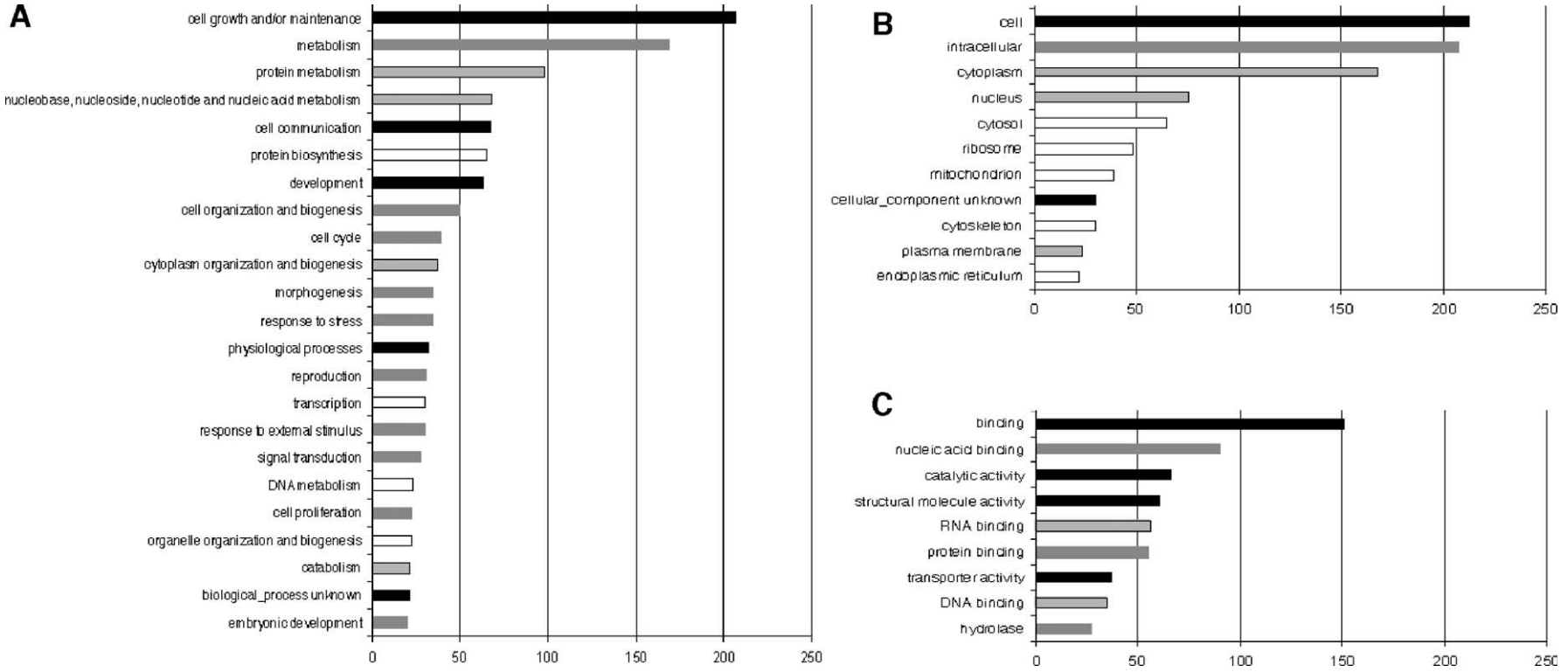

Fig. 4. Gene ontology terms described one fourth of the "early elongation" array. Biological processes (A), cellular components (B) and molecular functions (C) identified within the 230 unique sequences of the array, which were found in the Bos taurus gene Index (BtgI) with a link to a G.O. term (223 EST in panel A, 220 in B and 230 in C). In A, all the terms are shown but in B and C, only those representing more than 20 cDNA are illustrated. Terms listed at indent 2 in the Gene Ontology tree are given in black, those at indent 3 in grey, and those at indent 4 in white. Each additional indentation provides additional description but redundant information since one cDNA is often affected to several G.O. terms when its function is highly detailed in the literature. In addition, the "early elongation" array contained 443 non-overlapping sequences which were either identified but not annotated (270) or un-identified (173).

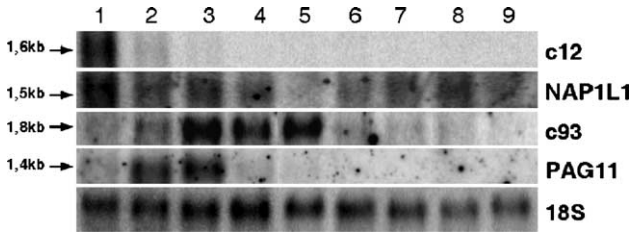

Fig. 5. Differential expressions during the elongation of bovine blastocysts. Northern blots with 4 cDNA out of the 8 statistically identified as differential between ovoid, tubular and early filamentous stages (lanes 1–3). Two cDNA displayed a preferential expression at the ovoid stage (c12, Nap1L1) and the others an increased expression at the early filamentous stage (c93 and PAG11). The c93 cDNA was also expressed after implantation (chorion 24 dpi, term villosity, lanes 4–5). Only c93 and Nap1L1 cDNAs were weakly expressed in adult somatic tissues such as intestine, lung, mammary gland or muscle (lanes 6–9). 18S RNA was hybridized as a positive control.

embryos under appropriate conditions for cross-species hybridization (data not shown).

Discussion

The aim of our work was to provide information on the proliferation and early differentiation of the trophoblast lineage in late-implanting cattle embryos during in vivo development. Our results define a developmental stage, the ovoid blastocyst, and a window of development (2 to 3 days in the bovine species) during which committed trophoblast cells, while still expressing epiblast specifying genes, co-express transcription factor and effector genes which initiate extra-embryonic differentiation and relate to cell proliferation processes. Two new genes were also identified for which, up to now, no equivalents have been found in the mouse. In light of these results, we at present believe in a bovine-specific programming of trophoblast development.

Differences between mouse and bovine trophoblast lineages

Morphological specificities of the ruminant trophoblast were already known (see the introduction) but molecular peculiarities in the expression patterns of lineage-specific genes had not previously been reported, except for the Oct-4 transcript. In our study, this mRNA was detected in the trophoblast of bovine spherical blastocysts (7 dpi) by whole-mount in situ hybridization and RT-PCR (Figs. 1 and 2), confirming data reported on the mouse where Oct-4 mRNA was found in the trophectoderm at 3.5 dpc (Rosner et al., 1990). However, an initial difference appeared at the spherical stage: the presence of Fgf4 mRNA in the trophectoderm of spherical bovine blastocysts. This transcript, which encodes a trophoblast growth factor secreted by the ICM, is restricted to the ICM in mouse blastocysts (Rappolee et al., 1994). Another difference appeared one stage later when Oct-4 transcripts were found for the first time in the trophoblast from ovoid blastocysts by 2 of the 3 techniques used here (Figs. 1 and 2). Oct-4 mRNA and protein have indeed been reported in the trophoblast of expanded blastocysts from several ruminants (cow, goat) as well as in pig (van Eijk et al., 1999; Kirchhof et al., 2000; He et al., 2005), while in one study (Kurosaka et al., 2004), no evidence of Oct-4 transcript was found. The Oct-4 protein seems to be restricted to the ICM lineage of early elongating bovine embryos (Vejlsted et al., 2005). The detection of Oct-4 mRNA in the trophoblast of such blastocysts suggests that either un-translated Oct-4 transcripts do not disappear quickly in bovine trophoblast cells or tiny amounts of Oct-4 protein are there but undetectable by immunohistochemistry on blastocyst sections.

Three other molecular features argued for differences between mouse and bovine trophoblast cells after gene-specific amplifications: the faint amount of Sox2 transcripts found in spherical and ovoid trophoblasts, the identification of two Nanog variants and the detection of a Gata-6 mRNA at the same stages. Based on the assumption that lineage specification and maintenance is achieved by the coordinate expression of positive and negative regulators in each cellular subset (Rossant, 2004), our data argue for a late restriction in the expression of epiblast and endoderm genes to their own lineages. Since recent reports have shown that the pluripotencial competence associated with Oct-4 or Nanog activities is a

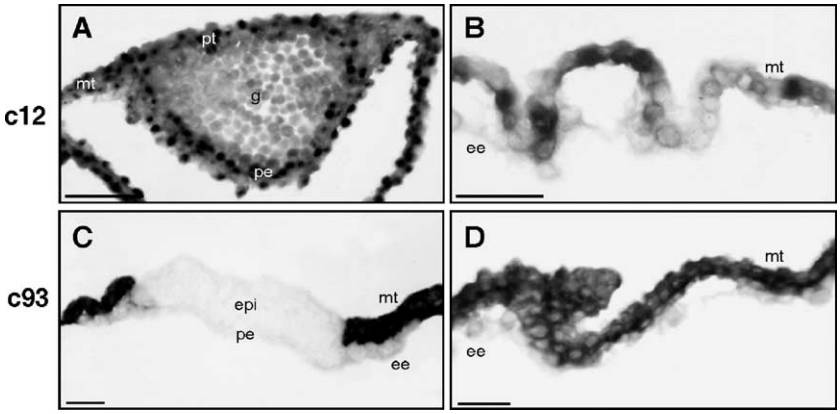

Fig. 6. Two new bovine genes with contrasted cellular expressions at the onset of elongation. In situ hybridization on blastocyst sections with c12 (A, B) and c93 (C, D) RNA probes at the ovoid (A), tubular (B) and early filamentous stages (C, D). Sense probes did not give any signal. Each pattern was established on 3 blastocysts per stage and 4 to 6 sections per blastocyst. Scale bar: 50 μm. g: germ disc, pe: primitive endoderm, pt: polar trophoblast, mt: mural trophoblast, ee: extra-embryonic endoderm, epi: epiblast, pe: primitive endoderm.

dose-dependant phenomenon (Niwa et al., 2000; Hatano et al., 2005), the presence of bovine Oct-4 and Nanog transcripts in the trophoblast suggests that their expression actively contributes to the maintenance of a "non-differentiating trophoblast" phenotype compatible with the intense cellular multiplication reported at the onset of elongation. We gathered indirect arguments for such an early proliferative phenotype based on both the expression of transcription factors which maintain the proliferation of trophoblast stem cells within the mouse polar lineage, such as Eomes and Elf5, and on the identification of genes known to be proliferation markers in human cancers: Nap1L1 and Opn (Lei et al., 2005). Based on mouse work (Avilion et al., 2003), the faint expression of Sox2 encoding transcripts in the bovine trophoblast could also argue for a delayed commitment to differentiate at both spherical and ovoid stages.

That such Oct-4 and Nanog expression levels favor a slow differentiating phenotype in the bovine trophoblast is an interesting hypothesis. As in mouse embryos, where Cdx2 and Oct-4 are concomitantly expressed at the late morula stage (Strumpf et al., 2005), the expression of Oct-4 and Nanog does not interfere with the expression of trophoblast-specific genes at the spherical and ovoid stages. Indeed, we identified transcripts encoding Cdx2, Hand1, Ets2 or IFN-tau in the bovine trophoblast at both spherical and ovoid stages. Conversely, one could suggest that the expression of the Oct-4 and Nanog genes in the trophoblast lineage results from a delayed or inefficient restriction of their expression to the epiblast due to a weak expression level of the trophoblast specifying factor, Cdx2.

Our data on the mural trophoblast lineage remains to be verified through protein localization, in vivo or in vitro functional studies. Such work is beyond the scope of this paper and appropriate tools must be adapted to the bovine model or entirely developed. For instance, no antibody is available against the bovine proteins analyzed here (http://www.biocompare.com) and cross reactivity with human or mouse tools requires a case by case evaluation. While in the mouse model gene networks involved in the establishment and/or maintenance of primary lineages can be examined using cultured cell lines (Chew et al., 2005; Hyslop et al., 2005; Ralston and Rossant, 2005), the same type of comparative studies cannot be run in the bovine model due to the lack of truly pluripotent ES or TS cells (TS for Trophoblastic Stem cells). Since in vivo RNA interference is not very effective for studies beyond the one cell stage (Hernandez and Bueno, 2005), we consider in vitro interference as a more appropriate approach since bovine trophoblastic cell lines have recently been characterized (Talbot et al., 2000; Ushizawa et al., 2005).

Differences between mouse and bovine trophoblast differentiation

Conversely to mouse trophoblast cells which differentiate into giant cells or ectoplacental cells after implantation, bovine trophoblast cells grow and differentiate through a specific elongation process prior to implantation. Accordingly, transcription factors which favor trophoblast differentiation and placenta formation, such as Hand1 or Ets2, are mainly expressed after implantation in the mouse but well ahead of implantation in bovine extra-embryonic tissues. Similar observations have also been reported for the Ets2 protein on the ovine trophoblast (Ezashi et al., 2001) and the Hand1 transcript on the human trophoblast (Knofler et al., 2002). Moreover, for two new transcripts isolated from the bovine cDNA array no orthologue has thus far been identified in the mouse either by sequence analysis or in situ hybridization. Among them, c12 was of special interest due to its strong expression at the onset of elongation, its reciprocal pattern to Oct-4, Nanog and Sox2 in the germ disc at the ovoid stage and its discontinuous expression in the mural trophoblast at the tubular stage. Such a discontinuous pattern has not been documented thus far for any transcript isolated from early elongating blastocysts in ruminants (sheep: Cammas et al., 2005; cattle: Ushizawa et al., 2004) or in pig (Ross et al., 2003; Blomberg et al., 2005; Lee et al., 2005). However, such a nuclear location has recently been recognized for natural Sense-Antisense Transcripts or SAT, as a result of mouse transcriptome studies, with a potential role in the regulation of gene expression (Kiyosawa et al., 2005). In addition, the local extinction of the c12 transcript in some cells of the tubular trophoblast is also to be considered since the local extinction of IFN-tau expression has been observed at places where the trophoblast comes into contact with the endometrium at the onset of the implantation phase (Xavier et al., 1997). What determines the local extinction of the c12 transcript at the tubular stage has now to be studied.

The ovoid stage is a critical step for trophoblast proliferation and differentiation

Along with the expression patterns of ten lineage specification genes from the mouse embryo, a new bovine transcript strongly expressed at the onset of the elongation process (c12) and about 900 unique sequences defining part of what the early elongation repertoire could be, we have presented new molecular data which argue for an important role of the ovoid stage during the blastocyst growth and differentiation. Indeed, the persistent expression of epiblast (Oct-4, Sox2, Nanog) and endoderm (Gata-6) specifying genes, the detection of proliferation markers (Opn, Nap1L1) and the expression of trophoblast-specific genes (Cdx2, Hand1, Ets-2, IFN-tau, c12) at the ovoid stage prompts us to consider it as an essential transition in polar and mural trophoblast development (Fig. 7). At this stage, mural trophoblast cells express both pluripotency and proliferation markers supporting the view of an intense multiplication of undifferentiated trophoblastic cells before differentiation become effective. Whether the maintenance of such a mural proliferation requires (bovine) specific processes different from those involved in the maintenance of trophoblast stem cells proliferation is highly probable, due to (i) the limited contacts of mural cells with the ICM and (ii) the relative low level of Eomes or Elf5 transcripts in the bovine mural

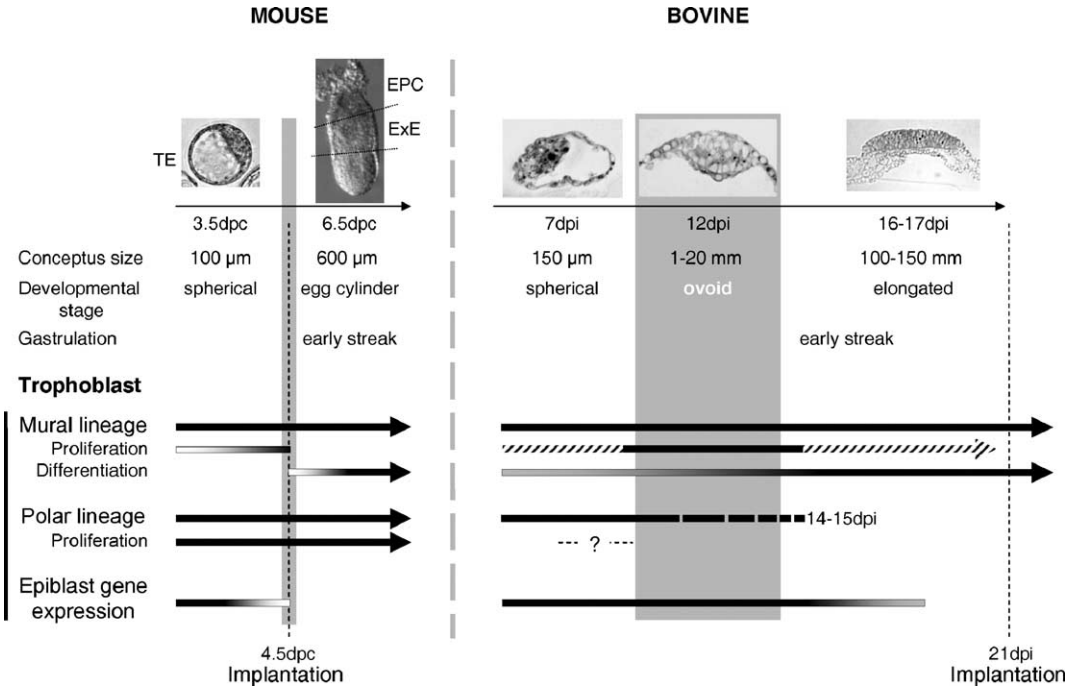

Fig. 7. Schematic view of developmental differences between bovine and mouse trophoblast lineages. In mouse blastocysts, the mural trophoblast does not proliferate that much and differentiates rapidly in primary trophoblast giant cells. The intense proliferation of the trophoblast is restricted to the polar lineage and stimulated by the ICM (3.5 dpc) or the epiblast (6.5 dpc). This proliferation depends on the maintenance of trophoblast stem cells through the expression of Eomes (polar TE) or Elf5 (ExE). Epiblast specifying genes, expressed in the trophoblast at 3.5 dpc, are restricted to the ICM at 4.5 dpc (Oct-4). In bovine blastocysts, the polar trophoblast (or Rauber's layer) has totally disappeared by 14 to 15 dpi. The lineage which grows and differentiates (or elongates) is the mural lineage. Epiblast specifying genes (Oct-4, Sox2, Nanog) are transcribed in trophoblast cells of spherical and ovoid blastocysts and Nanog transcripts are also found at the filamentous stage. These transcripts could maintain a slow differentiating phenotype and favor an intense cell multiplication at the onset of elongation. Whether this proliferation is restricted to the ovoid stage and depends on (bovine) specific processes has to be investigated. Photographs of mouse blastocysts (3.5 dpc and 6.5 dpc) were kindly provided by Dr. A. Jouneau. End processes are represented by bars, ongoing processes by arrows, appearing or disappearing functions by graded colors, hypothetical functions by hatched lines, unknown features by question marks.

trophoblast as compared to their expression in the mouse polar lineage (Russ et al., 2000; Donnison et al., 2005).

Moreover, the identification at that stage of genes involved in cell migration (Opn), epithelium organization (DAB-2) and trophoblast/epiblast interactions (Furin) seems essential to us with regard to what happens between the spherical and the tubular stages, namely: inner cell mass/epiblast transition, primitive endoderm formation and migration, as well as the onset of new blastocyst shapes (ovoid, tubular, filamentous). Recent reports have shown that the molecular signs of gastrulation do, in fact, precede the formation of the antero-posterior axis and the anatomical identification of the primitive streak (Idkowiak et al., 2004; Rodriguez et al., 2005). Considering physiological data indicating that most embryonic losses observed in the field after insemination occur by day 14 or earlier (Dunne et al., 2000), we propose that the ovoid stage is also critical for blastocyst survival due to both the decisive developmental steps occurring at that time for further blastocyst development and the way the developing blastocyst has to cope with the signals emitted by the uterus at the onset of elongation (Gray et al., 2002; Imakawa et al., 2004).

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