Morphological characterization of pre- and peri-implantation *in vitro* cultured, somatic cell nuclear transfer and *in vivo* derived ovine embryos

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Abstract

The processes of cellular differentiation were studied in somatic cell nuclear transfer (SCNT), *in vitro* cultured (IVC) and *in vivo* developed (*in vivo*) ovine embryos on days 7, 9, 11, 13, 17 and 19. SCNT embryos were constructed from *in vitro* matured oocytes and granulosa cells, and IVC embryos were produced by *in vitro* culture of *in vivo* fertilized zygotes. Most SCNT and IVC embryos were transferred to recipients on day 6 while some remained in culture for day 7 processing. *In vivo* embryos were collected as zygotes, transferred to intermediate recipients and retransferred to final recipients on day 6. All embryos were processed for examination by light and transmission electron microscopy or immunohistochemical labelling for alpha-1-fetoprotein and vimentin. Overall, morphological development of *in vivo* embryos was superior to IVC and SCNT embryos. Day 7 and particularly day 9 IVC and SCNT embryos had impaired hypoblast development, some lacking identifiable inner cell masses. On day 11, only *in vivo* and IVC embryos had developed an embryonic disc, and gastrulation was evident in half of *in vivo* embryos and one IVC embryo. By day 13, all *in vivo* embryos had completed gastrulation whereas IVC and SCNT embryos remained retarded. On days 17 and 19, *in vivo* embryos had significantly more somites and a more developed allantois than IVC and SCNT embryos. We conclude that IVC and particularly SCNT procedures cause a retardation of embryo development and cell differentiation at days 7–19 of gestation.

Reproduction (2005) 130 681–694

Introduction

Since the birth of the first lamb cloned from an adult somatic cell (Campbell *et al.* 1996, Wilmut *et al.* 1997), the technique of somatic cell nuclear transfer (SCNT) has been applied to a wide range of animal species. It is, however, evident that the process is associated with a dramatic loss of embryos and fetuses throughout gestation, with the majority being lost in the first trimester (Hill *et al.* 2000). In particular, the period of implantation appears to be critical (Wilmut *et al.* 1997, Cibelli *et al.* 1998, de Sousa *et al.* 2001). SCNT embryos display retarded embryonic development and a variety of organ malformations, as well as an increased frequency of the large offspring syndrome (LOS), manifesting itself as increased birth weight and increased gestation length for pregnancies brought to term (Wilson *et al.* 1995, Young *et al.* 1998, de Sousa *et al.* 2001, Chavatte-Palmer *et al.* 2004). Examinations of placental tissues from SCNT pregnancies have revealed

deviant placental vascularization and development, a phenomenon that results in increased occurrence of hydrallantois and the presence of enlarged placentomes (Hill *et al.* 1999, Heyman *et al.* 2002). In addition, abnormal placental formation plays a crucial role in the development of LOS and is a major cause of embryo and fetal loss (Hill *et al.* 2000, Bertolini & Anderson 2002). The causes of pathological placental development have not been fully elucidated, but numerous studies on gene expression (Daniels *et al.* 2000, Wrenzycki *et al.* 2001, 2002, Humpherys *et al.* 2002) have revealed disproportionate expression patterns in cloned embryos, supporting the hypothesis that epigenetic reprogramming of SCNT embryos is abnormal (Humpherys *et al.* 2001, Young *et al.* 2001, Dean *et al.* 2003, Han *et al.* 2003, Shi *et al.* 2003).

Proper placental formation requires several developmental processes. Sheep placentation is chorioallantoic and is initiated with the budding of the allantois from the endoderm of the hind gut. Subsequently, placental

vascularization is formed by the visceral mesoderm surrounding the allantois. Hence, a proper formation of the germ layers (endoderm, mesoderm and ectoderm) during the process of gastrulation is a key requirement for normal placental development.

In this investigation, we included three experimental groups: *in vivo* embryos as a control, *in vitro* cultured (IVC) embryos cultured in a medium supplemented with 20% human serum in order to provoke embryonic stress leading to LOS changes (Thompson *et al.* 1995, Walker *et al.* 1996a), and SCNT embryos.

The present study aimed to examine IVC and SCNT embryos in order to determine whether placental abnormalities previously associated with these procedures may have their origins during differentiation of the germ layers and formation of the allantoic bud. Pre- (days 7, 9, 11 and 13) and peri-implantation (days 17 and 19) ovine embryos produced by *in vitro* culture or SCNT were compared with *in vivo* derived embryos. All embryos were submitted to stereo-microscopy, and embryos collected on days 7, 9, 11 and 13 were processed for either transmission electron microscopy (TEM) or immunohistochemical examination of germ layer formation with the markers alpha-1-fetoprotein and vimentin (Maddox-Hyttel *et al.* 2003).

Materials and Methods

Overall experimental design

Three groups of embryos were analysed: *in vivo* derived embryos (*in vivo* embryos), *in vivo* fertilized but *in vitro* cultured embryos (IVC embryos), and somatic cell nuclear transfer embryos (SCNT embryos). *In vivo* and IVC embryos were fixed (see later) on days 7, 9, 11, 13, 17 and 19 after fertilization, and SCNT embryos on the same days after nuclear transfer.

Preparation of ewes

Donor ewes

Oestrus was synchronized by the administration of an intravaginal progestagen pessary (flugestone acetate; 45 mg; Laboratoire Pharmaceutique Porges, Paris, France) for 12 days. Superovulation was induced with follicle-stimulating hormone (FSH) (10 ml, i.m.; Folltropin, Vetrepahm, Ontario, Canada) administered in six decreasing doses commencing 48 h before pessary removal and thereafter at 12-h intervals. Pregnant mare serum gonadotrophin (PMSG, 500 IU i.m. Pregnecol; Horizon Technology, North Ryde, NSW, Australia) was administered concurrently with the initial FSH injection. At 12 h after the final FSH injection, gonadotropin-releasing hormone (GnRH, 0.3 ml i.m.; Fertagyl, Intervet International, Boxmeer, Netherlands) was administered. Donors were artificially inseminated directly into the uterine lumen with a laparoscope, with approximately 1×10^7 motile sperm 14 h after GnRH treatment. Semen was collected

from rams of proven fertility and diluted with PBS containing 5% heat inactivated sheep serum (HISS). The day of ovulation was designated as day 0.

Recipient ewes

Oestrus was synchronized as for donor ewes with a progestagen pessary. PMSG (0.4 ml i.m.) was administered at the time of pessary removal. All recipients were synchronized within ± 12 h of donor ewes.

Generation of embryos

All reagents were obtained from Sigma and were of cell culture grade unless otherwise specified.

In vivo embryos

Twelve ewes were used to generate *in vivo* embryos. Putative zygotes were recovered from the oviducts of superovulated donors and transferred (10–20 per ewe) directly to the oviducts of synchronized intermediate recipients ($n = 2$). Embryos were subsequently recovered on day 6, and resultant blastocysts transferred (10–15 per ewe) to final recipients ($n = 20$) for recovery on days 7, 9, 11, 13, 17 and 19. For recovery of embryos, ewes were killed by a lethal dose of phenobarbitone (Virbac Australia Pty Ltd, Peakhurst, New South Wales, Australia), and the intact reproductive tract was excised. Once each tract had been trimmed of excess tissue, PBS with 5% HISS was flushed through the uterine horns in a retrograde direction from the uterotubal junction. Fluid passing out through the body of the uterus was collected into 90 mm Petri dishes. Embryos were identified under a stereo-microscope and fixed as detailed below.

In vitro cultured (IVC) embryos

Putative zygotes were recovered from superovulated donor ewes and placed directly into pre-equilibrated modified synthetic oviduct fluid (mSOF) medium (Tervit *et al.* 1972) supplemented with 20% heat-inactivated human serum (HIHS). Subsequent culture was conducted at 38.5 °C in a humidified atmosphere of 5% O₂, 5% CO₂ and 90% N₂. On day 6, the majority of resultant blastocysts were transferred to synchronized recipients ($n = 8$), as described above (10–15 blastocysts per ewe), and subsequently recovered from the reproductive tract on days 9, 11, 13, 17 and 19. The remaining blastocysts were cultured to day 7 and then fixed directly from the culture medium.

Somatic cell nuclear transfer (SCNT) embryos

SCNT embryos were constructed according to previously described protocols, using oocytes from adult ovaries for cytoplasm production and adult granulosa cells as karyoplasts (Peura 2003).

Karyoplast derivation

Granulosa cells were obtained by removing cumulus cells from immature *in vivo* sourced oocytes and culturing them to passage 3 in Glasgow's medium (Gibco, Life Technology, Auckland, New Zealand) supplemented with sodium pyruvate, L-glutamine, DMEM, penicillin, streptomycin and 5% heat-inactivated fetal calf serum (FCS) (CSL, Parkville, Australia). Once cells were 90% confluent, they were frozen in a 10% DMSO and 90% FCS solution in Nunc cryotubes (Nunc, Roskilde, Denmark) at a concentration of approximately 200 000 cells/ml and stored in liquid nitrogen. On the day of use, a cryotube of frozen cells was thawed in a 45 °C water bath, washed with fresh Glasgow's medium and centrifuged (2500 r.p.m., 20 min) to remove cryoprotectants. The cell pellet was then resuspended with modified Glasgow's medium and held at 38.5 °C until used for cell injection (within 1–4 h).

Cytoplasm production

Briefly, ovaries were collected from a local slaughterhouse. Follicles of >2 mm were aspirated, and the cumulus–oocyte-complexes (COCs) were placed in HEPES-buffered TCM-199 (Sigma) supplemented with 2% HISS and 100 i.u./ml heparin. COCs were washed in and then transferred to four-well plates (Nunc; 25 per well) containing 0.6 ml maturation medium (TCM-199; Sigma) supplemented with 20% HISS; 5 µg/ml FSH, 5 µg/ml luteinizing hormone (LH) and 1 µg/ml oestradiol. The medium was overlaid with mineral oil (0.3 ml), and culture occurred at 38.5 °C in a humidified atmosphere of 5% CO₂ in air. Cumulus cells were removed from COCs 18 h later with hyaluronidase (500 i.u./ml) and pipetting. Denuded oocytes were placed in mSOF supplemented with 4 mg/ml BSA (Gibco Life Technologies) and amino acids at ovine oviduct fluid concentrations (Walker *et al.* 1996b) until enucleated. As required, groups of oocytes (maximum 20) were incubated in hSOF (HEPES-buffered SOF with 4 mg/ml BSA) containing 7.5 µg/ml cytochalasin B and 5 µg/ml Hoechst (33342) for 5 min and then placed in enucleation medium (hSOF containing 7.5 µg/ml cytochalasin B). Enucleation involved the removal of a minimal amount of cytoplasm from the area directly underlying the polar body. All material removed from the oocyte was viewed under UV light to confirm that the metaphase II plate had been removed. After enucleation, the karyoplast was immediately injected into the perivitelline space through the enucleation hole in the zona pellucida. After reconstruction, cytoplasm–karyoplast couplets were held in culture media until the time of fusion.

Fusion

Nuclear fusion was performed with a Genaust Fusion Machine (Genetics Australia, Bacchus Marsh, Australia) in a mannitol fusion medium (0.3 M mannitol, 0.05 mM CaCl₂ and 0.1 mM MgSO₄). The manipulation chamber comprised two parallel, 0.1 mm diameter platinum wires

separated by 0.2 mm. An AC-alignment pulse of 400 kHz and 10 V was followed by two DC pulses of 1250 V/cm and 80 µs. Fusion was assessed after 30 min, and unfused couplets were re-pulsed. Fused couplets were placed in 10 µM Ca²⁺-ionophore in protein-free hSOF for 5 min, before incubation in 2 mM 6-DMAP for a minimum of 2 h. Finally, reconstructed embryos were placed in 0.6 ml mSOF overlaid with mineral oil and cultured in a humidified atmosphere of 5% O₂, 5% CO₂ and 90% N₂ at 38.5 °C.

Embryo development

Cellular cleaving was recorded on day 2 and blastocyst development on day 6. Day 7 blastocysts were recovered directly from the culture medium and fixed. Good-quality blastocysts (and a small number of compacted morulae) scheduled for collection on days 9–19 were vitrified on day 6 by the open pulled straw method (Vajta *et al.* 1996). These embryos were subsequently transferred to synchronized recipients (*n* = 20) after thawing. Only embryos without apparent cell lysis in the inner cell mass and presenting a reformed blastocoele cavity were transferred. Up to 24 blastocysts were transferred per ewe, except for ewes designated for recovery on days 17 and 19, where only up to nine embryos were transferred. Embryos were recovered from recipients on days 9, 11, 13, 17 and 19 and fixed.

Embryo fixation and processing

After recovery, embryos were fixed for either TEM or immunohistochemistry. For TEM, embryos were fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.2) for 1 h at 4 °C, and then transferred to 0.1 M PB and transported from Australia to Denmark in 0.1 M PB with 3 mg/ml polyvinyl alcohol (PVA). Post-fixation took place in 1% 0.1 M PB followed by dehydration and embedding in Epon (Hyttel & Madsen 1987). Embryos for immunohistochemistry were fixed in 4% paraformaldehyde (PFA) in 0.1 M PB (pH 7.2) for 1 h at 4 °C and then transferred to 1% PFA in 0.05 M PB. Embryos were transported in 1% PFA before dehydration and embedding.

After initial fixation, embryos were submitted to stereo-microscopy and digital image processing. A total of 52 *in vivo*, 38 IVC and 62 SCNT embryos were analysed in this way (Table 1). Subsequently, the embryos or parts thereof, including the embryo proper, were embedded in agar blocks for further processing. When only the embryo proper was processed, the material was orientated in the blocks to produce sagittal sections through the embryo proper. Days 7, 9, 11 and 13 embryos were submitted to sectioning and further analysis.

Transmission electron microscopy (TEM)

Epon-embedded embryos were serially cut into semi-thin sections (2 µm) that were stained with 1% (w/v) toluidin

Table 1 Embryo diameter and length, length of embryonic disc, length of embryo proper, width of allantois and number of somites in ovine *in vivo*, IVC and SCNT embryos at different stages of development. Data on hatching status are also shown for days 7 and 9. Figures are indicated as mean $\pm$ s.d. Figures with different superscripts within rows are significantly different ($P < 0.05$, Student's *t*-test on mean value).

	<i>In vivo</i>	IVC	SCNT
Day 7			
Number	6	6	8
Diameter, embryo (μm)	318 $\pm$ 55 ^{a*}	344 $\pm$ 97 ^{a*}	160 $\pm$ 7 ^{b*}
Zona pellucida intact (number)	0	1	6
Hatching (number)	1	4	1
Hatched (number)	5	1	1
Day 9			
Number	6	7	6
Diameter, embryo (μm)	950 $\pm$ 314 ^{a**}	433 $\pm$ 172 ^{b**}	175 $\pm$ 50 ^{b**}
Zona pellucida intact (number)	0	1	1
Hatching (number)	0	0	3
Hatched (number)	6	6	2
Day 11			
Number	6	6	13
Length, embryo (mm)	2.7 $\pm$ 0.6 ^a	2.9 $\pm$ 1.3 ^a	0.8 $\pm$ 0.3 ^b
Width, embryonic disc (μm)	328 $\pm$ 79 ^a	214 $\pm$ 50 ^b	120 $\pm$ 17 ^c
Day 13			
Number	9	6	13
Length, embryo (mm)	46.3 $\pm$ 16.9 ^a	38.1 $\pm$ 14.3 ^a	2.8 $\pm$ 3.2 ^b
Width, embryonic disc (μm)	962 $\pm$ 236 ^a	798 $\pm$ 291 ^a	293 $\pm$ 172 ^b
Day 17			
Number	14	5	10
Length, embryo proper (mm)	6.20 $\pm$ 0.7 ^a	5.6 $\pm$ 2.0 ^a	5.6 $\pm$ 1.0 ^a
Width, allantois (mm)	9.3 $\pm$ 5.2 ^a	6.7 $\pm$ 7.0 ^{ab}	3.8 $\pm$ 1.7 ^b
Somites (number)	19.1 $\pm$ 3.8 ^a	14.2 $\pm$ 3.6 ^b	13.3 $\pm$ 3.1 ^b
Day 19			
Number	11	8	12
Length, embryo proper (mm)	11.5 $\pm$ 1.8 ^a	6.7 $\pm$ 1.3 ^b	7.3 $\pm$ 1.6 ^b
Width, allantois (mm)	70.6 $\pm$ 21.9 ^a	12.3 $\pm$ 5.0 ^b	13.7 $\pm$ 7.8 ^b
Somites (number)	27.8 $\pm$ 3.4 ^a	21.4 $\pm$ 0.9 ^b	22.5 $\pm$ 4.2 ^b

* Figures based on zona pellucida intact and hatched, but not hatching embryos.

** Figures based on hatched embryos.

blue and analysed by bright-field light microscopy (LM). A total of 41 embryos were analysed; 11 *in vivo*, 12 IVC and 19 SCNT embryos. Selected sections were re-embedded in Epon (Hyttel & Madsen 1987), cut into ultrathin sections (70 nm) and stained with uranyl-acetate/lead citrate. TEM was performed on a Phillips CM100 transmission electron microscope, and digital image processing was applied.

Immunohistochemistry

Serial sections (4 μm) of each embryo were cut. Selected sections were stained with haematoxylin-eosin and examined by LM for evaluation and orientation. A total of 51 paraffin-wax-embedded embryos were evaluated. Of these, 17 *in vivo*, 13 IVC and 20 SCNT embryos were selected for immunohistochemistry (Table 2). Remaining sections were kept at 4 °C until further use.

Consecutive paraffin sections were labelled with two immunohistochemical markers to visualize germ layer formation (Maddox-Hyttel *et al.* 2003). The chosen primary antibodies were rabbit polyclonal anti-human alpha-1-fetoprotein for potential endoderm and monoclonal mouse anti-human vimentin for potential mesoderm. Sections were collected on Super Frost ++ slides

(Mentzel GmbH + Co KG, Braunschweig, Germany) and allowed to adhere overnight, before being dewaxed and rehydrated. Subsequently, the slides were submitted to antigen retrieval by microwave treatment (3 $\times$ 5 min) in sodium citrate buffer (0.01 M; pH 6.0). Endogenous peroxidase activity was blocked by 3% (v/v) H₂O₂ in

Table 2 Numbers of *in vivo*, IVC and SCNT embryos presenting cells with positive staining for alpha-1-fetoprotein and vimentin at different stages of development. Total number of analysed embryos is indicated in parentheses.

	<i>In vivo</i>	IVC	SCNT
Day 7			
Alpha-fetoprotein	0 (4)	0 (3)	0 (4)
Vimentin	0 (4)	0 (3)	0 (4)
Day 9			
Alpha-fetoprotein	0 (3)	0 (4)	0 (2)
Vimentin	0 (3)	0 (3)	0 (2)
Day 11			
Alpha-fetoprotein	1 (5)	0 (3)	0 (8)
Vimentin	1 (4)	1 (3)	1 (7)
Day 13			
Alpha-fetoprotein	2 (4)	2 (3)	0 (6)
Vimentin	5 (5)	3 (3)	3 (6)

methanol, and non-specific binding of antibodies was avoided by protein blocking agent (protein block, DAKO, Glostrup, Denmark). Incubation with the primary antibodies was performed overnight at 4 °C (alpha-1-fetoprotein: 1:5000 (DAKO); vimentin: 1:2500 (Zymed, Carlton Court, San Francisco, USA) diluted in PBS with 1% BSA). Throughout the procedures, negative control tissue was included with every slide and incubated overnight with non-immune sera (for alpha-1-fetoprotein: rabbit IgG (DAKO); vimentin: mouse IgG1 (DAKO)). Positive control tissue (paraffin-embedded bovine day 21 embryos (Maddox-Hyttel *et al.* 2003)) was included with every staining batch. The primary antibodies were visualized by DAKO LSAB + kit followed by AEC substrate kit (Zymed), and specimens were counterstained with Mayer's haematoxylin (1 min). Finally, the sections were mounted with DAKO permanent aqueous mountant.

Results

Stereo-microscopical characterization

The kinetics of hatching as well as measurements of embryonic diameter, embryonic length, embryonic disc length, allantois width, embryo proper length and somite numbers are presented in Table 1. In general, the embryos collected at days 7 and 9 were spherical blastocysts; day 11 embryos were larger and some clearly ovoid, whereas a combination of spherical, ovoid and tubular embryos was

collected on day 13. In the ovoid and tubular embryos, an embryonic disc (ED) could, in most instances, be identified. On days 17 and 19, all analysed embryos displayed a clearly identifiable embryo proper.

The IVC embryos and, in particular, the SCNT embryos were slower in hatching than the *in vivo* embryos (Table 1). At day 7, the diameter of the SCNT embryos was significantly smaller than that of the *in vivo* and IVC embryos (Fig. 1), whereas at day 9, both the hatched SCNT and IVC embryos were smaller than the hatched *in vivo* embryos. At day 13, the SCNT embryos were shorter than the IVC and *in vivo* embryos, and there was an associated reduction in the size of their ED (Fig. 2). At day 17, no significant differences between the three groups of embryos were noted with respect to the length of the embryo proper. However, the width of the allantois as well as the number of somites was greater in the *in vivo* embryos than in the IVC and SCNT embryos. At day 19, the length of the embryo proper, the width of the allantois and the number of somites were greater in *in vivo* embryos than in IVC and SCNT embryos (Fig. 3).

General morphology

The general morphological description is based on evaluation of paraffin and Epon sections by bright-field light microscopy and of Epon sections by TEM.

At day 7, five of six *in vivo* embryos presented a well-defined inner cell mass (ICM), and three had initiated the

Figure 1 Stereo-micrographs of typical day-7 embryos. (a) *In vivo* embryo devoid of a zona pellucida. (b) IVC embryos, two of which are devoid of their zona pellucida. Note the darker appearance of these embryos compared with 'a'. (c) SCNT embryos, some of which are in the process of hatching.

Figure 2 Stereo-micrographs of day-11 and day-13 *in vivo* embryos, illustrating size and stage of development. (a) Ovoid day-11 embryo during initial elongation. The embryonic disc (ED) is clearly visible. (b) Elongated day-13 embryo with prominent, protruding embryonic disc (c).

formation of the hypoblast (Fig. 4). Consequently, the remaining cells of the ICM are referred to as the epiblast. The hypoblast initially appeared directly beneath the epiblast and proceeded along the inside of the trophoblast, but none of the embryos had a complete hypoblast lining. At the ultrastructural level, the epiblast cells were uniform and rounded, presenting large nuclei and prominent nucleoli and a cytoplasm dominated by mitochondria, polyribosomes and lipid droplets. The hypoblast cells were squamous and linked by tight junctions. The trophoblast cells were particularly rich in intermediate filaments, some of which engaged in the formation of desmosomes, along with which tight junctions were noted.

Among the IVC embryos, four of six presented a well-defined ICM, and three of these had initiated hypoblast formation. In two embryos, no ICM or epiblast was identified,

although an incomplete hypoblast was observed in one. Cellular debris was noted in the perivitelline space of unhatched and hatching embryos. Only one of eight SCNT embryos presented a well-defined ICM and epiblast. This embryo displayed initial formation of the hypoblast; this, however, was also noted in another embryo in which no epiblast was identified. In the unhatched and hatching embryos, abundant accumulations of cellular debris in the peri-vitelline space was evident (Fig. 4).

At day 9, four of six *in vivo* embryos presented a well-defined epiblast. All *in vivo* embryos had a complete hypoblast lining the inside of the trophoblast; in two embryos, the hypoblast directly beneath the epiblast had assumed a more cuboidal appearance. At the ultrastructural level, Rauber's layer, while still intact, appeared as a thin layer of cytoplasmic trophoblast processes.

Figure 3 Stereo-micrographs of day-19 embryos. (a) *In vivo* embryo. The red line illustrates the measurement of the allantois (AL). Note the embryo proper (EP) and the rudimentary yolk sack (YS). The largest of the day-19 embryos: *in vivo* embryo (b), IVC embryo (c) and (d) SCNT embryo. The red line illustrates the measurement of the embryo proper. The smallest of the day-19 embryos: (e) *in vivo* embryo, (f) IVC embryo and (g) SCNT embryo.

In four of seven IVC embryos, a well-defined ICM or epiblast was found, while six embryos presented a complete hypoblast. At the ultrastructural level, IVC embryos displayed features comparable with day 7 *in vivo* embryos. Only one of the six SCNT-embryos presented a well-defined ICM or epiblast. This embryo also had an incomplete hypoblast. A complete and an incomplete hypoblast were, however, also noted in two embryos without an apparent epiblast.

At day 11, five of six *in vivo* embryos presented a well-defined ED consisting of an apparently pseudostratified or,

in some regions, bilayered epiblast with its underlying hypoblast (Fig. 5). The hypoblast extended into a complete lining of the trophoctoderm. Evidence of mitotic activity was observed primarily in the upper layer of the epiblast, and dome-shaped epiblast protrusions were seen at the apical epiblast surface. The hypoblast cells directly beneath the epiblast were cuboidal. At the ultrastructural level, the epiblast cells appeared to have formed a well-defined superficial layer of cells that displayed microvilli and were connected by putative tight junctions (Fig. 6). Intercellular junctions were not observed elsewhere in the

Figure 4 Light micrographs of typical day-7 embryos. (a) *In vivo* embryo showing a well-defined epiblast (E) and a developing hypoblast (H). (b) IVC embryo in the process of hatching. Note the debris in the perivitelline space (arrowhead) peripheral to the trophectoderm (arrow). (c) SCNT embryo with intact zona pellucida. Note the abundant debris in the perivitelline space (arrowhead) peripheral to the trophoblast (arrow).

epiblast. At the transition from superficial epiblast to trophoblast, putative tight junctions were observed between these two cell types. In three embryos, evidence of initial gastrulation appeared as a single cluster or small clusters of cells in the space between the epiblast and hypoblast. These putative endoderm and mesoderm precursor cells were rounded or slightly cuboidal and presented a large nucleus, prominent nucleoli and mitochondria as dominant features. The precursor cells made contact to each other through short cytoplasmic processes.

All the six IVC embryos presented a smaller but well-defined ED and a complete hypoblast, whereas initial gastrulation was not yet evident (Fig. 5). Signs of a pseudostratified or bilayered organization of the epiblast were noted in only three of six IVC embryos. In seven of 13 SCNT embryos, an epiblast was found together with a

complete hypoblast, but signs of a pseudostratified or bilayered organization of the epiblast were evident in only a single embryo. Among the six embryos without an ICM or epiblast, five presented a complete hypoblast. At the ultrastructural level, the epiblast of the SCNT embryos resembled the earlier (day 9) *in vivo* epiblasts. Remnants of Rauber's layer were seen frequently along the surface of the apical epiblast. The hypoblast cells directly beneath the epiblast were not as cuboidal or as closely apposed as in the *in vivo* and IVC embryos, and initial gastrulation was not observed in any of the SCNT embryos.

At day 13, all nine *in vivo* embryos displayed a well-defined ED presenting a well-developed ectoderm and endoderm as well as mesoderm, of which the extra-embryonic parts were clearly divided into visceral and somatic portions (Fig. 7). The upper ectoderm cells presented marked dome-shaped protrusions and numerous

Figure 5 Light micrographs of typical day-11 embryos. (a) *In vivo* embryo showing large, well-defined epiblast (E) with underlying hypoblast (H). Note the pseudostratified organization of the epiblast and the initial development of mesoderm (M) in the space between epiblast and hypoblast. (b) IVC embryo showing a smaller but still well-defined epiblast (E) and a complete hypoblast (H). Signs of initial mesoderm (M) formation are noted. (c) SCNT embryo displaying a small but well-defined epiblast (E). A complete hypoblast (H), but no mesoderm formation is noted.

Figure 6 Transmission electron micrographs from the superficial portion of the epiblast in day-11 *in vivo* embryo. (a) The superficial epiblast cells (E) form a tight layer. (b) Detail of framed section in (a) enlarged. Adjacent epiblast cells are connected by putative tight junctions (TJ).

mitoses. In three embryos, amniotic folds were present as uni- or bilaterally trophectoderm upfoldings in close proximity to the ectoderm. At the ultrastructural level, the ectoderm cells resembled their epiblast precursors on day 11 *in vivo* embryos. Again, mesoderm cells made contact with each other through cytoplasmic processes.

All IVC embryos had a clearly identifiable ED and all displayed endoderm and mesoderm formation, but visceral and somatic components of the latter were only clearly evident in two of six embryos. No evidence of amniotic folds was seen. In nine of 13 SCNT embryos, a clearly identifiable ED was observed. In six embryos, initial gastrulation was noted, but none had progressed to form visceral and somatic mesoderm, or had initiated amnion formation.

Immunohistochemistry

Immunohistochemical staining was performed on paraffin sections for alpha-1-fetoprotein and vimentin (Table 2).

Alpha-1-fetoprotein

Staining was confined to the cytoplasm of hypoblast cells. Weak staining was observed in a single, day-11 *in vivo* embryo. Evident staining was seen in *in vivo* and IVC embryos on day 13, whereas none of the SCNT embryos exhibited staining.

Vimentin

All *in vivo* embryos displayed a positive cytoplasmic staining for vimentin in the ventral part of the epiblast/ectoderm and in all components of the mesoderm on day 13 (Fig. 8). A single embryo, without evidence of gastrulation, showed staining in the ventral portion of the epiblast on day 11. All IVC embryos displayed staining of the ventral epiblast/ectoderm on day 13 and, if present, in the mesoderm. On day 11, one of three embryos stained positive in the ventral portion of the epiblast. Among the SCNT embryos, three of six day-13 embryos showed positive staining of a few cells in the ventral parts of the epiblast (Fig. 8). Similar staining was noted in a single embryo on day 11.

Discussion

In the present study, both pre- and peri-implantation development of the manipulated (IVC and SCNT) sheep embryos was assessed. *In vivo* embryos were used as a control. IVC embryos were cultured in a medium sup-

plemented with 20% human serum in order to provoke embryonic stress leading to LOS changes (Thompson *et al.* 1995, Walker *et al.* 1996a).

The stereo-microscopic measurements clearly showed that *in vivo* embryos, with respect to the size of the embryo, the length and development of the embryo proper, and the size of the allantois, consistently developed at a faster rate than SCNT embryos and, to a lesser degree, IVC embryos. The magnitude of this effect is probably even more pronounced than what appears from the presented data, as embryos that were most impaired after the period of *in vivo* development in the recipient were potentially eliminated from the IVC and SCNT groups. Moreover, the variation in developmental measures was much more pronounced among IVC embryos and, in particular, SCNT embryos than among their *in vivo* counterparts, a phenomenon that would render only the most advanced IVC and SCNT individuals likely to continue development to term (Holm *et al.* 1996, Koo *et al.* 2002). Sheep embryos containing low cell numbers have been shown to display decreased survival rates and increased sensitivity to asynchrony in the uterine environment (Willadsen 1981). Furthermore, the degree of DNA fragmentation is increased in SCNT embryos, mainly in the ICM cells, indicating that SCNT embryos with low cell numbers have a higher rate of apoptosis (Fahrudin *et al.* 2002). Both the increased variation in embryo size and observed delay in development could thus be expected to have damaging effects on the continued embryonic growth of the IVC embryos and, particularly, the SCNT embryos.

During the pre- and peri-implantation development, embryos of the large domestic species undergo three sequential rounds of differentiation where cells are recruited from an initially totipotent but, later, pluripotent cell population. At the first round of differentiation, the terminally differentiated trophoblast and the pluripotent ICM are formed, while the second round of differentiation results in delamination of the hypoblast from the ICM, a process after which the latter cell population remains pluripotent and is referred to as the epiblast. Gastrulation represents the third round of differentiation where the basic germ layers are formed by a complex process including establishment of the primitive streak and involution of cells serving as precursors of endoderm and mesoderm. The endoderm cells are inserted into the upper portion of the hypoblast lining, whereas the mesoderm develops into embryonic and extra-embryonic portions, of which the latter was divided into somatic and visceral parts. Marked differences between the three different groups of embryos were noted with respect to the differentiation of the ICM

Figure 7 Light micrographs of typical day-13 *in vivo* and SCNT embryos. (a) *In vivo* embryo displaying organization of the trophectoderm (Te), ectoderm (Ec), endoderm (En) and mesoderm, of which the extra-embryonic portion is organized into somatic mesoderm (SM) and visceral mesoderm (VM). (b) Detail from (a) showing the multilayered ectoderm (Ec), the mesoderm (M) and the endoderm (En). (c) SCNT embryo displaying involution of cells from the epiblast (E) for mesoderm (M) and putative endoderm (En) formation. (d) Detail from (c) showing the epiblast (E), mesoderm, (M) and putative endoderm (En).

Figure 8 Light micrographs of vimentin-stained, day-13 embryos. (a) *In vivo* embryo displaying positive staining in the ventral portion of the ectoderm (Ec) and in the somatic (SM) and visceral mesoderm (VM), whereas the endoderm (En) is unstained. (b) IVC embryo displaying positive staining of the ventral portion of the epiblast (E). (c) SCNT embryo displaying weak positive staining in only a few epiblast cells (E).

into hypoblast and epiblast, particularly in the timing of this event. On day 7, hypoblast and epiblast were seen in all three groups of embryos, though at a higher frequency among the *in vivo* embryos. On day 9, all *in vivo* embryos and most of the IVC embryos had formed a complete hypoblast, which was, on the other hand, observed in only a single SCNT embryo. Interestingly, this embryo did not possess a recognizable epiblast. This was also noted in several of the IVC and SCNT embryos on days 9 and 11 respectively. As the hypoblast is formed by the delamination of ICM cells (Wintenberger-Torrès & Fléchon 1974, Maddox-Hyttel *et al.* 2003), it seems likely that, in some cases, the formation of the hypoblast consumes the complete cell population contained in the ICM. This observation renders hypoblast formation a predominant event in the developing blastocyst. A proportion of the IVC embryos and, in particular, the SCNT embryos also lacked an identifiable ICM before the differentiation into hypoblast and epiblast. Such embryos have probably been impaired even earlier and may have contained sufficient viable cells only to form the trophoblast during the first round of differentiation. The presence of a functional ICM would not have been easily recognized before transfer (van Soom *et al.* 1997, McEvoy *et al.* 2000), and hatching of blastocysts devoid of an evident ICM has been reported in cattle (Avery *et al.* 1995). Thus, trophoblast differentiation may be a developmental event similar in predominance to hypoblast formation. Such so-called trophoblastic vesicles have experimentally been shown to be capable of maintaining intrauterine growth and elongation in the sheep, thus enabling the persistence of such vesicles (Heyman *et al.* 1984, Fléchon *et al.* 1986).

Large differences were noted between the experimental groups with respect to the process of gastrulation. In half of the *in vivo* embryos, initial gastrulation was noted on day 11, whereas this event was only in its very early beginning in a single IVC embryo and was not seen at all in SCNT embryos. In agreement with earlier findings in the sheep (Guillomot *et al.* 2004), mesoderm and endoderm precursors were present before any morphological evidence of primitive streak formation. Gastrulation is preceded by the formation of a pseudostratified or bilayered organization of the epiblast, as has earlier been reported in farm animals (Betteridge & Fléchon 1988, Flechon *et al.* 2004), a phenomenon which was, in general, delayed by approximately 2 days in IVC embryos and, in particular, SCNT embryos. When the timing of gastrulation was assessed with immunohistochemical markers, a similar delay was observed, especially in SCNT embryos.

IVC and SCNT embryos also displayed delay with respect to the formation of the extra-embryonic membranes, including the amnion and the allantois, which are crucial for continued development. In the large domestic species, the amnion is formed by budding of the trophoblast into chorionic folds (Bryden *et al.* 1972, Maddox-Hyttel *et al.* 2003). This process was initiated in half of the *in vivo* embryos on day 13, whereas none of the IVC and SCNT embryos exhibited amniotic folds. Allantois formation marks an important stage of development when the embryonic portion of the future chorioallantoic placenta is prepared. On day 17, but particularly on day 19, the width of the allantoic bud was greater in *in vivo* embryos than among their IVC and SCNT counterparts. Placental formation is a process demanding a certain synchrony between embryonic (the allantois and associated

vascularization) and maternal (the endometrium) components, and the impaired allantoic development may well form the basis of the placental deviations noted later during SCNT-based gestation (Hill *et al.* 1999, Heyman *et al.* 2002). Furthermore, a deviant placenta could cause perinatal deaths in SCNT offspring brought to term (Chavatte-Palmer *et al.* 2004).

In conclusion, SCNT and IVC embryos develop slower than *in vivo* embryos, a phenomenon that may have detrimental effects on placentation, thus partly explaining why high embryo and fetal loss is associated with these techniques.

Acknowledgements

We thank Ms Hanne Holm and Jytte Nielsen, of the Royal Veterinary and Agricultural University, Frederiksberg, Denmark, for their outstanding assistance in the preparation and processing of the embryos, and Louise Engberg, DVM, and Camilla Rasmussen, DVM, for their precise stereo-microscopical classifications. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 18 June 2005

First decision 21 July 2005

Revised manuscript received 29 July 2005

Accepted 19 August 2005