Somatic cell nuclear transfer alters peri-implantation trophoblast differentiation in bovine embryos

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Abstract

Abnormal placental development limits success in ruminant pregnancies derived from somatic cell nuclear transfer (SCNT), due to reduction in placentome number and consequently, maternal/fetal exchange. In the primary stages of an epithelial–chorial association, the maternal/fetal interface is characterized by progressive endometrial invasion by specialized trophoblast binucleate/giant cells (TGC). We hypothesized that dysfunctional placentation in SCNT pregnancies results from aberration in expression of genes known to be necessary for trophoblast proliferation (Mash2), differentiation (Hand1), and function (IFN- τ and PAG-9). We, therefore, compared the expression of these factors in trophoblast from bovine embryos derived from artificial insemination (AI), *in vitro* fertilization (IVF), and SCNT prior to (day 17) and following (day 40 of gestation) implantation, as well as TGC densities and function. In preimplantation embryos, Mash2 mRNA was more abundant in SCNT embryos compared to AI, while Hand1 was highest in AI and IVF relative to SCNT embryos. IFN- τ mRNA abundance did not differ among groups. PAG-9 mRNA was undetectable in SCNT embryos, present in IVF embryos and highest in AI embryos. In postimplantation pregnancies, SCNT fetal cotyledons displayed higher Mash2 and Hand1 than AI and IVF tissues. Allelic expression of Mash2 was not different among the groups, which suggests that elevated mRNA expression was not due to altered imprinting status of Mash2. The day 40 SCNT cotyledons had the fewest number of TGC compared to IVF and AI controls. Thus, expression of genes critical to normal placental development is altered in SCNT bovine embryos, and this is expected to cause abnormal trophoblast differentiation and contribute to pregnancy loss.

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Introduction

Somatic cell nuclear transfer (SCNT) has been employed to produce clones of a wide variety of mammalian species since the first successful use of differentiated donor cells was reported (Wilmut *et al.* 1997). However, the success of cloning by nuclear transfer has not come without complication. In the 10 years of SCNT research, the efficiency of this technique has not improved. Several elegant studies have been conducted to increase the proportion of reconstructed embryos that develop to blastocysts and determine altered gene expression compared to IVF blastocysts (for review, see Heyman 2005, Wrenzycki *et al.* 2005). Unfortunately, fewer than 6% of the reconstructed bovine blastocysts develop into viable offspring (Hill *et al.* 2000a). From the time of embryo transfer (day 8) to day 90 of gestation, 80% of

SCNT pregnancies are lost (Hill *et al.* 2000b). A major problem associated with the mortality of these reconstructed embryos is poor placental development (Hill *et al.* 2000b, De Sousa *et al.* 2001, Ono *et al.* 2001a, 2001b, Ogura *et al.* 2002).

Some of the most common placental anomalies associated with SCNT pregnancies in ruminants are a reduced number of placentomes, poor vascular development, hydroallantois, enlarged placentomes, and increased fetal membrane weights in the later stages of gestation (Wells *et al.* 1999, Hill *et al.* 2000b, Hashizume *et al.* 2002). Abnormal placental development has also been reported earlier in gestation, during the critical time of early postimplantation. Hill *et al.* (2000b) observed reduced vascularity and epithelial height in the placentomal regions of SCNT pregnancies at days 45–55. In addition, day 60 SCNT bovine

pregnancies had less organized fetal villi development and fewer placentomes compared to AI pregnancies (Hashizume *et al.* 2002). These defects suggest aberrant developmental processes in SCNT placentas.

As with other species of mammals, the first cellular differentiation of the bovine embryo occurs with the formation of the blastocyst, resulting in the inner cell mass and trophoblast cells that will develop into the embryo proper and extra-embryonic tissues, respectively (McLaren 1990). As pregnancy ensues, ruminant trophoblast cells proliferate and differentiate into either mononucleate or binucleate/giant cells (Wooding & Wathes 1980). The mononucleate population comprises the majority of the trophoblast contribution to the placenta, and these cells are involved in the production of interferon- τ (IFN- τ), the pregnancy recognition signal in cattle (Roberts *et al.* 1992). The trophoblast binucleate/giant cells (TGC), which makes up approximately 20% of the trophoblast population, migrate across the fetomaternal junction and fuse with uterine epithelial cells (Wooding & Wathes 1980, Klisch *et al.* 1999). These TGC undergo acytokinetic mitosis to become tetraploid, and may also undergo endoreduplication, with a resultant DNA content that can be as high as 32N (Klisch *et al.* 1999). These cells produce pregnancy-related proteins, such as pregnancy-associated glycoproteins (PAGs), placental lactogen (PL), and prolactin-related proteins, all are detectable in the maternal serum as pregnancy progresses (Wooding 1981, Kessler & Schuler 1991, Zoli *et al.* 1992).

Reports on TGC populations in SCNT placentas have ranged from fewer (Hashizume *et al.* 2002), no difference (Hoffert *et al.* 2005), or more (Ravelich *et al.* 2004a) when compared to IVF and AI placentas from early gestation (days 30–60). Even though these results are in conflict, they clearly indicate that trophoblast development in SCNT embryos is altered. In addition, the mRNA and protein levels for gene associated with TGC function such as PL and PAG vary between naturally derived and SCNT placentas (Hashizume *et al.* 2002, Patel *et al.* 2004b, Ravelich *et al.* 2004b, Hoffert *et al.* 2005). Given the morphological variation and consequent dysfunction of TGC in placentas from SCNT embryos, it is of considerable interest to explore the expression of factors involved in bovine trophoblast differentiation during the early stages of placental formation.

Most of the work till date has been focused on the expression of bovine genes at the blastocyst stage where experimental samples are more readily available. It is highly possible that many genes, which regulate later stages of trophoblast development, are not expressed at this stage. Moreover, while there are numerous morphological descriptions of bovine TGC, there are few studies in gene regulation during bovine TGC differentiation. Further, the majority of the genes driving trophoblast differentiation in the cow is not known, so investigators had to rely on the information gleaned from other

species. Gene inactivation studies in mice have identified two genes that are essential to trophoblast differentiation: mammalian achaete-scute complex homologue-like 2 (Mash2; also known as Ascl2; Guillemot *et al.* 1994) and heart and neural crest cell derivative 1 (Hand1; Cross *et al.* 1995). Both genes are basic helix-loop-helix transcription factors, and appear to have opposing activities (Cross *et al.* 1995). In the mouse, Mash2 stimulates cell proliferation and inhibits progression of trophoblast to its terminally differentiated giant cell form (Cross *et al.* 1995, Tanaka *et al.* 1997, Rossant *et al.* 1998), whereas Hand1 evokes giant cell formation (Riley *et al.* 1998). Mash2 homozygous mutant mouse embryos, which are lost during mid-gestation period (day 9.5 postcoitum) have excessive number of trophoblast giant cells (Guillemot *et al.* 1994). Embryos from mice with mutated Hand1 have reduced numbers of giant cells and pregnancy is also lost during mid-gestation period (Riley *et al.* 1998). In the cow, we have shown Mash2 to be expressed specifically in the placenta, and in greatest abundance during the time of trophoblast proliferation prior to implantation (Arnold *et al.* 2006). As in the mouse (Guillemot *et al.* 1995), bovine Mash2 is an imprinted gene with the paternal allele silenced after implantation (Arnold *et al.* 2006).

The goal of the present study was to examine these genes of known significance to trophoblast differentiation and function during the pre- and early postimplantation periods of the cow and to determine how their expression correlates with the TGC population in placental tissues of AI, IVF, and SCNT produced embryos.

Materials and Methods

Animals

All animal treatment protocols were approved by the Comité de déontologie, Faculté de médecine vétérinaire, Université de Montréal in accordance with regulations of the Canadian Council of Animal Care. Mature (>2 years of age) Holstein cows were chosen as recipients.

Oocyte collection and in vitro maturation (IVM)

Oocytes obtained from slaughterhouse ovaries were matured *in vitro* as previously described (Bordignon *et al.* 2003). Briefly, cumulus-oocyte complexes (COC) were aspirated from 2 to 7 mm follicles and washed in HEPES-buffered tissue culture medium (TCM)-199 (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (FBS). Only COCs with several layers of cumulus cells and with homogenous oocyte cytoplasm were selected. Groups of 25 COCs were cultured in 100 μ l drops of IVM media (bicarbonate-buffered TCM-199 supplement with 10% FBS, 50 μ l/ml luteinizing hormone (LH) (Ayerst, London, Ontario, Canada), 0.5 μ g/ml follicle-stimulating hormone (FSH) (Follitropin-V;

Vetrepharm, St-Laurent, PQ, Canada), 1 µg/ml estradiol-17β (Sigma), 22 µg/ml pyruvate (Sigma), and 50 µg/ml gentamicin (Sigma)). After 20–22 h of IVM, matured COCs were randomly assigned to either IVF or SCNT groups.

Production of somatic cell nuclear transfer embryos (SCNT)

Cumulus cells were removed from oocytes with a 0.2% hyaluronidase (Sigma) solution and oocytes with the first polar body present were selected for nuclear transfer as previously described (Bordignon *et al.* 2003). Briefly, cumulus-denuded oocytes were placed in PBS containing 7.5 µg/ml cytochalasin B (Sigma) and approximately 30% of the host cytoplasm adjacent to the first polar body was removed. To confirm the removal of chromatin, host oocytes were placed in medium containing 5 µg/ml Hoechst 33342 (Sigma) for 15 min and subjected briefly to u.v. irradiation.

A primary fibroblast cell line from a *Bos taurus* × *Bos indicus* male fetus collected at 60 days of gestation, was used for SCNT. Donor cells were cultured in Dulbecco's modified Eagles medium supplemented with 10% FBS and 50 units/ml penicillin–streptomycin (Invitrogen) at 39 °C in a humidified atmosphere of 5% CO₂. Donor cultures between two and five passages were allowed to progress to confluence and maintained for 2 days prior to use so cells would be in the G1/G0 stage of the cell cycle. A single fetal fibroblast cell was introduced into the perivitelline space of the enucleated oocyte. The resulting couplet was placed in a 0.3 M mannitol solution containing 0.1 mM MgSO₄ and 0.05 mM CaCl₂ and subjected to a 1.5 kV electric pulse lasting 70 µs. Couplets were then washed and placed into 50 µl drops of modified synthetic oviductal fluid (mSOF: 108 mM NaCl, 7.2 mM KCl, 0.5 mM MgCl₂, 2.5 mM NaHCO₃, 1.7 mM CaCl₂·H₂O, 0.5 mM glucose, 0.33 mM pyruvic acid, 3 mM lactic acid, 8 mg/ml BSA, 150 µg/ml gentamicin, 0.01% phenol red, 1.4 mM glycine, 0.4 mM alanine, 1 mM glutamine, 2% essential amino acids, and 1% non-essential amino acids; Gardner *et al.* 1994). After 1–2 h in culture, couplets were examined to determine fusion and exposed to 5 mM ionomycin (Sigma) for 4 min to induce parthenogenetic activation. Reconstructed oocytes were cultured in mSOF at 39 °C in a humidified atmosphere of 5% CO₂ and 5% O₂ for 7 days.

Production of in vitro (IVF) and in vivo embryos (AI)

For IVF embryos, matured COCs were fertilized as previously described (Parrish *et al.* 1986) and cultured in mSOF at 39 °C in a humidified atmosphere of 5% CO₂ and 5% O₂ for 7 days. For AI embryos, Holstein cows were either injected with 500 µg cloprostenol (Estrumate, Schering-Plough Animal health, Pointe-Claire, Quebec, Canada) to induce estrus and artificially inseminated or

subjected to a superovulation protocol as previously described (Price *et al.* 1999). Briefly, cows were given i.m. injections of Follitropin-V (Vetrepharm) every 12 h in decreasing doses starting at days 9–10 of the estrous cycle (day 0 = estrus). Cows then received an injection of 500 µg cloprostenol at 52 h and were artificially inseminated at 86 h after the initiation of superovulation respectively. Semen used for AI and IVF was from the same *Bos indicus* bull used to produce the day 60 fetal donor cells.

Production and collection of day 17 embryos and day 40 tissues

On day 8 of *in vitro* culture, blastocyst stage SCNT and IVF embryos were non-surgically transferred into synchronized Holstein cows. For day 17 embryo collections, 10–12 embryos were transferred per recipient. For day 40 samples, 1–2 embryos were transferred per recipient. At day 17 of pregnancy, filamentous embryos were collected from recipients or superovulated cows (AI group) by non-surgical flushing of the uterus with sterile PBS + 0.4% BSA. Embryos were snap-frozen and stored at –70 °C until further processed. For day 40 samples, pregnancy was confirmed via ultrasonography, animals were slaughtered and pregnant uteri with viable fetuses were collected at the abattoir and transported on ice to the laboratory. For each gravid uterus, intercotyledonary tissue and four cotyledons were collected and fixed with 10% buffered formalin. The remaining cotyledons were snap-frozen and stored at –70 °C until further processed.

RNA extraction, purification, and reverse transcriptase (RT) reaction

Individual day 17 embryos were homogenized and RNA was purified using TRIzol LS Reagent (Invitrogen) as recommended by the manufacturer. Day 40 tissues were homogenized in buffer RLT (Qiagen) with 0.12 M β-mercaptoethanol (Sigma) and RNA was purified using an RNeasy Protect Mini kit (Qiagen), as recommended by the manufacturer. Total RNA was measured by spectrophotometry at 260 nm and 1.0 µg/sample of total RNA was used for the RT reaction using the Moloney murine leukemia virus (M-MLV) RT (Promega Biosciences, Inc.) according to manufacturer's instructions.

Quantitative RT-PCR

For the analysis of relative mRNA abundance, bovine-specific primers for Mash2 (GenBank Accession No. DQ333219), IFN-τ (GenBank Accession No. AF270471), and PAG-9 (GenBank Accession No. AF020511) were developed (Table 1). Bovine-specific primers for glyceraldehyde 3-phosphate dehydrogenase (Gapdh; GenBank Accession No. AF077815) were used as an internal control.

Table 1 Oligonucleotide primer sequences used for reverse transcriptase-PCR.

Gene	Primer (5'-3') sense and antisense	Length (bp)	Annealing temperature (°C)
Gapdh	TGTTCCAGTATGATTCCACCC TCCACCACCTGTTGCTGTA	791	58
Hand1	GCTCTCCAAGATCAAGACTCTGC CGGTGCGTCCTTTAATCCTCTTC	224	58
IFN- τ	GCTATCTCTGTGCTCCATGAGATG AGTGAGTTTCAATCTCCACCCATC	353	58
Mash2	CGCTGCGCTCGGCGGTTGAGTA GGGACCCGGGCTCCGAGCTGTG	210	67.5
PAG-9	TCCTTTTGTACCATGCCAGC TGCCCTCCTGCTTGTTTTG	330	58

Hand1 primers (Table 1) were designed based on the homologous sequences between the human (GenBank Accession No. NM004821) and mouse (GenBank Accession No. NM008213; Table 1). PCR products of the expected size were excised and purified using a Gel Extraction kit (Qiagen). Purified cDNA was then ligated into a pGEM-T Easy Vector System I (Promega Corp.) according to manufacturer's instructions, and further transformed into competent *Escherichia coli* strain XL-1 blue. Plasmids were isolated by the use of a QIAprep Spin Miniprep kit (Qiagen) and sequenced using a ABI PRISM 310 sequencer (Applied Biosystems, Foster City, CA, USA), and at least three independent samples were sequenced for the verification of authenticity. Sequences were analyzed against known homologous Hand1 sequences and submitted to GenBank (Accession No. DQ381724).

Steady-state amounts of mRNA were analyzed using specific quantitative RT-PCR (qRT-PCR) assays (Itoh *et al.* 1998). Briefly, PCR products using gene-specific primers (Table 1) were gel extracted and pooled using Qiaquick Gel Extraction and QIAquick Purification kits respectively (Qiagen) to generate standard curves ranging from 0.01 to 100 fg/ μ l. Five known standard concentrations and samples were subjected to PCR amplification consisting of an initial denaturing (95 °C/5 min), cycles of denaturing (94 °C/30 s), annealing (primer-specific temp/30 s) and elongation (72 °C/30 s), and a final elongation (72 °C/4 min). Optimal cycle number for amplification during the exponential phase was determined for each gene. Cycle numbers for day 17 samples were: Gapdh, 19; Mash2, 27; Hand1, 27; IFN- τ , 15; and PAG-9, 40. For day 40 cotyledonary tissue, 20, 35, and 26 cycles were used for Gapdh, Mash2, and Hand1 respectively. PCR products were analyzed with SYBR Safe (Invitrogen) and calculated with a computer imaging system utilizing the Macintosh NIH software (National Institute of Health, Bethesda, MD, USA).

Determination of parental allele expression of Mash2

To determine the parental allele expression of Mash2, a PCR–restriction fragment length polymorphism (PCR–RFLP) assay was utilized as previously described (Arnold

et al. 2006). Briefly, a 210 bp Mash2 PCR product from *Bos taurus* (BT) and *Bos indicus* (BI) cotyledonary tissues were excised, purified, and sequenced as described earlier. A single polymorphism was detected (T/C) between the BT and BI sequences. The polymorphism was within the recognition site of the restriction enzyme SfiI, which allowed the paternal allele (BI) to be digested (giving two bands of 135 and 75 bps), whereas the maternal allele (BT) remained intact (Fig. 4A). PCR products from day 17 and day 40 AI, IVF, and SCNT samples were digested with SfiI at 50 °C for 15 h.

Determination of trophoblast binucleate/giant cells density and function

To determine the proportion of TGC in the trophoectoderm, formalin-fixed cotyledonary tissues were embedded in paraffin and sectioned at 4 μ m. Slides were stained by periodic acid-Schiff (PAS) and counterstained with hematoxylin to aid in the identification of multinucleate giant cells as previously described (Klisch *et al.* 1999). For each slide, cells were counted from five randomly selected microscope fields at 400 $\times$ magnification. Cell counting was performed using a procedure with animal and treatment unknown to the examiner.

To confirm TGC numbers and function, a monoclonal antibody, SBU-3 (kindly provided by Garry Barcham, University of Melbourne, Victoria, Australia) raised against sheep trophoblast microvillous preparation that recognizes ruminant binucleate cells (Lee *et al.* 1985) was used. The same antibody has been used previously to characterize binucleate cell distribution in bovine placentomes (Lee *et al.* 1986). After washes in PBS, sections were incubated with a CY3 fluorochrome-labeled secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Nuclei were detected with 4',6-diamidino-2-phenylindole (DAPI) diluted 1:1000. Control sections were subjected to the same procedure except the SBU-3 antibody was replaced with 5% BSA in PBS. For each slide, nuclei and positive SBU-3 cells were counted from eight randomly selected areas (at 400 $\times$ magnification). Cell counting was performed using a procedure with animal and treatment unknown to the examiner.

Statistical analysis

The ratio of target gene/*Gapdh* (fg/fg) was used as a value for each sample and data were analyzed using the least square ANOVA by the general linear model procedures of SAS. The proportions TGC and positive SBU-3 cells were analyzed using the least square ANOVA by the general linear model procedures of SAS. Duncan's multiple range test was utilized to analyze values when significant differences in groups were found. A probability level of $P < 0.05$ was defined as significant.

Results

Quantification of *Mash2*, *Hand1*, *INF- τ* , and *PAG-9* mRNA at day 17

To evaluate if the expression of trophoblast differentiation genes are altered in SCNT preimplanted bovine embryos, the relative abundance of *Mash2* and *Hand1* mRNA was compared among AI ($n=6$), IVF ($n=6$), and SCNT ($n=8$) day 17 embryos. In addition, *INF- τ* and *PAG-9* mRNA were evaluated to determine the trophoblast cell function among all the groups. To adjust for individual variation among samples, all genes were analyzed with reference to the housekeeping gene *Gapdh*. The relative abundance of *Gapdh* mRNA was similar for all groups (Fig. 1). The abundance of *Mash2* mRNA was 2.5-fold greater in SCNT embryos than AI ($P < 0.05$), whereas IVF embryos were not different among the three groups ($P > 0.10$; Fig. 2A). Opposite of *Mash2*, *Hand1* mRNA abundance in AI and IVF embryos were 2.5-fold greater than SCNT ($P < 0.05$, Fig. 2A). Abundance of *INF- τ* mRNA did not differ among groups (Fig. 2B), suggesting normal mononucleate cell function among all groups. The abundance of *PAG-9* mRNA was undetectable in SCNT embryos, and twofold lower in the IVF group compared to AI ($P < 0.05$, Fig. 2B), indicating an alteration in TGC number or function in SCNT embryos. These results suggest abnormal or

Figure 1 Histogram of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) mRNA from artificial insemination (AI, black), *in vitro* produced (IVF, white), and somatic cell nuclear transfer (SCNT, grey) day 17 embryos and day 40 cotyledonary tissues.

Figure 2 Relative abundance of mRNA of genes for (A) trophoblast development (*Mash2* and *Hand1*) and (B) function (*INF- τ* and *PAG-9*) in day 17 AI (black), IVF (white), and SCNT (grey) embryos. Histograms represent fg/fg *Gapdh*. The quantification represents means $\pm$ S.E.M. of individual samples (AI and IVF, $n=6$; SCNT, $n=8$). Different superscripts represent significant differences in means ($P < 0.05$).

delayed trophoblast differentiation in embryos produced by SCNT.

Quantification of *Mash2* and *Hand1* mRNA at day 40

To determine if altered expression of *Mash2* and *Hand1* is normalized in SCNT embryos that survive past the time of implantation, cotyledonary tissues were collected from day 40 fetuses ($n=3$ per group). The relative abundance of *Mash2* and *Hand1* mRNA was highest in SCNT cotyledons compared to IVF and AI cotyledons ($P < 0.05$, Fig. 3). These results indicate that *Mash2* and *Hand1* expression remains altered in SCNT embryos even though they successfully attach and develop placental structures.

Parental allele expression of *Mash2*

A single polymorphism detected between *Bos taurus* and *Bos indicus* *Mash2* PCR products (Fig. 4A) was used to determine the parental origin of *Mash2* by PCR-RFLP. At day 17, expression of *Mash2* was found to be from both

Figure 3 Relative abundance of Mash2 and Hand1 mRNA in day 40 cotyledonary tissues from AI (black), IVF (white), and SCNT (grey) embryos. Histograms represent fg/fg Gapdh. The quantification represents means $\pm$ S.E.M. of individual samples ($n=3$ /group). Different superscripts represent significant differences in means ($P<0.05$).

paternal and maternal alleles regardless of group (Fig. 4B). However, the maternal allele appears to produce more Mash2 mRNA than the paternal allele (Fig. 4B). At day 40, the paternal Mash2 allele appears to be silenced, with a few embryos still expressing from both alleles, regardless of experimental group (Fig. 4C). These results indicate that the parental regulation of the bovine Mash2 gene is not altered in SCNT embryos.

Trophoblast giant cell density and function

To establish whether the altered gene expression of Mash2 and Hand1 correlates with modified trophoblast cell populations in bovine SCNT embryos, TGC densities and function were evaluated. Day 40 cotyledonary tissues from AI, IVF, and SCNT embryos were stained

Figure 4 Analysis of parental expression of Mash2 in day 17 embryos and day 40 cotyledonary tissues by PCR-RFLP. (A) Single polymorphism difference (grey box) between the maternal (*Bos taurus*; BT) and paternal (*Bos indicus*; BI) Mash2 PCR products and recognition sequence (white box) for SfiI restriction enzyme (arrow indicates cleavage site). Digestion of Mash2 PCR products from day 60 *Bos taurus*, *Bos indicus* mixed BT/BI (M) cotyledonary tissue and AI, IVF, and SCNT day 17 embryos (B) and day 40 cotyledonary tissue (C).

with PAS and counterstained with hematoxylin to determine the proportion of TGC in the trophoderm (Fig. 5). No difference was detected in the percentage of the TGC in AI (Fig. 5A) and IVF (Fig. 5C) cotyledonary tissue ($P>0.10$; Table 2). However, the number of TGC in SCNT cotyledonary tissue (Fig. 5E) was reduced compared to the AI and IVF groups ($P<0.05$; Table 2).

To confirm TGC populations and determine if TGC from SCNT cotyledonary tissues are functioning properly, the MAB to PAGs, SBU-3, was used. The SBU-3 antibody specifically labels trophoblast binucleate cells in placental region of ruminants (Lee *et al.* 1986). At day 40, cotyledonary tissue from AI fetuses had the highest percentage of positive cells (Fig. 5B; Table 2), followed by the IVF group (Fig. 5D; Table 2). Similar to the PAS staining, cotyledonary tissue from SCNT fetuses had the lowest proportion of positive cells (Fig. 5F; Table 2; $P<0.01$). These results suggest that there are fewer TGC in day 40 cotyledonary tissue from SCNT fetuses, but these cells are able to produce the TGC-specific protein, PAG.

Discussion

It has been well documented that cattle embryos produced by SCNT have abnormal placental development. These abnormalities are thought to be the major factors causing the high pregnancy loss associated with this procedure. Several studies have shown reduced number of placentomes and altered cellular organization of the SCNT placenta (Stice *et al.* 1996, Wells *et al.* 1999, Hill *et al.* 2000b, Hashizume *et al.* 2002). Till date much of the molecular research in SCNT pregnancies, past the blastocyst stage, has focused on the genes involved in the maternal/fetal interface, or those expressed by the TGC (Hashizume *et al.* 2002, Davies *et al.* 2004, Ravelich *et al.* 2004a, Hoffert *et al.* 2005, Oishi *et al.* 2006). In the present study, the focus was on genes believed to control development of the two principal trophoblast cell populations. In addition, the present study analyzes two time points during the preimplantation period that have been shown to be critical for successful placental development. The present data suggest that altered placental development of SCNT embryos may be due to the abnormal expression of genes that are crucial for normal trophoblast differentiation in the cow.

At day 17 of pregnancy in cattle, the trophoblast cells undergo rapid proliferation, resulting in elongation of the embryo throughout the uterus prior to attachment. It has been documented that the differentiation of the bovine trophoblast cells begins to occur during this time resulting in the production of the first TGC (Greenstein *et al.* 1958, Wathes & Wooding 1980, Wooding 1983). Ruminant TGC play a critical role in the initial attachment and development of the maternal/fetal interface, as seen in other species, such as the mouse

Figure 5 Immunohistochemical characterization of trophoblast binucleate/giant cells (TGC) in cotyledonary tissue from day 40 AI, IVF, and SCNT fetuses. Hematoxylin/PAS staining of AI (A), IVF (C), and SCNT (E) cotyledonary tissues. Positive TGC for SBU-3 (red) in AI (B), IVF (D), and SCNT (F) cotyledonary tissue. Nuclei counterstained with DAPI (blue). Bars = 100 μ m.

Table 2 Percentage of trophoblast giant cells and SBU-3 positive cells in day 40 cotyledonary tissue from embryos produced by artificial insemination (AI), *in vitro* fertilization (IVF), and somatic cell nuclear transfer (SCNT).

Group	Animal*	TGC/total cells (%)	SBU-3 positive/nuclei (%)
AI	8	403/2076 (19.4%)	604/4109 (14.7%)
	4	385/2110 (18.3%)	644/4426 (14.6%)
	2	482/2614 (18.4%)	598/3980 (15.0%)
	Group total	1270/6800 (18.7%) ^a	1846/12515 (14.8%) ^a
IVF	9	334/2076 (16.1%)	409/4235 (9.7%)
	1	354/1966 (18.0%)	358/3987 (9.0%)
	5	383/2240 (17.1%)	477/4482 (10.6%)
	Group total	1071/6282 (17.1%) ^a	1244/12704 (9.8%) ^b
SCNT	7	288/2079 (13.9%)	395/5283 (7.5%)
	6	219/1832 (12.0%)	187/3423 (5.5%)
	3	324/2145 (15.1%)	316/4628 (6.8%)
	Group total	831/6056 (13.7%) ^b	898/13334 (6.7%) ^c

a,b,c $P < 0.05$.

*Four cotyledons per animal were evaluated.

(Sutherland 2003) and human (Janatpour *et al.* 1999). Two genes that play vital role in trophoblast differentiation are Mash2 and Hand1. Both of these genes are basic helix-loop-helix transcription factors and appear to exert opposing influences on trophoblast differentiation (Cross *et al.* 1995). Mash2 stimulates trophoblast proliferation and inhibits TGC formation (Guillemot *et al.* 1995), whereas Hand1 stimulates TGC development (Riley *et al.* 1998). As in other species, Mash2 is a placenta-specific gene in the cow (Arnold *et al.* 2006). In the present study, abundance of Mash2 was higher in day 17 SCNT embryos, compared to *in vivo* derived embryos. Day 17 IVF embryos appear to produce intermediate levels of Mash2 mRNA compared to SCNT and AI embryos. These results suggest that *in vitro* culturing may contribute to the altered expression of Mash2 and these differences are amplified in embryos produced by SCNT. Several reports have demonstrated that *in vitro* culturing of bovine embryos causes altered placental development, as well as large offspring syndrome (for review, see Farin *et al.* 2004). The present results support the previous findings that Mash2 expression from day 8 blastocyst stage SCNT embryos, produced with G0/G1 donor cells, was elevated compared to IVF blastocyst embryos (Wrenzycki *et al.* 2001). Contradictory to the latter report, day 17 AI embryos had less Mash2 mRNA than IVF-produced embryos. These differences may be due to the stage of embryogenesis analyzed. In addition, the present study directly compared SCNT, IVF, and AI embryos and distinguished the differences due to SCNT or *in vitro* culturing and their ability to develop *in vivo* to day 17. These data indicate that SCNT embryos were unable to correctly express genes important to trophoblast development and *in vitro* culturing required to produce these embryos magnified these expression differences.

We have shown herein that expression of Hand1 is reduced in day 17 SCNT embryos compared to both AI

and IVF embryos. Hand1 has been shown to be required for the differentiation of trophoblast cells to giant cells in other species. In the mouse, Hand1 mutants arrest development around 7.5 days postcoitum and have significantly reduced number of trophoblast giant cells (Riley *et al.* 1998). In the cattle, expression of Hand1 mRNA is not detected until the embryonic ovid stage at day 12 postinsemination (Degrelle *et al.* 2005). The increase in Hand1 expression during this stage of development coincides with the differentiation of the trophoblast cells into TGC prior to implantation. This raises the possibility that the balance between the antipodal actions of Hand1 and Mash2 determines the differentiation of the trophoblast cell. Cross *et al.* (1995) reported that Rcho-1 trophoblast cell lines transfected to overexpress Hand1 differentiated into giant cells. Giant cell development was inhibited when Hand1-expressing Rcho-1 cells co-expressed Mash2 (Cross *et al.* 1995). The reduced abundance of Hand1 mRNA and the elevated abundance of Mash2 in SCNT day 17 embryos suggests limitation in the capacity of trophoblast cells to differentiate into TGC. Another potential explanation is that the overall development is delayed in SCNT embryos relative to that seen in *in vivo* derived embryos. Till date, evaluation of the developmental status of bovine SCNT embryos has relied primarily on overall appearance (i.e., days to reach blastocyst stage and trophoblast: inner cell mass ratios). If there is in fact a delay in expression of vital development genes, as our data suggest that this may cause asynchrony between the embryo and recipient. This factor, in itself, may explain the greater pregnancy losses associated with SCNT pregnancy. In contrast, pregnancy rates did not change when day 8 blastocyst stage SCNT embryos were transferred into recipient cows at day 7 of the estrous cycle (DR Arnold, unpublished observations). This further suggests that differences seen in the present experiment are due to altered expression of the genes of interest and not to delays in development.

To examine trophoblast cell function, evaluation of expression of genes known to be exclusive to mononucleate cells (IFN- τ) and TGC (PAG-9) was employed. IFN- τ , a secretory protein that inhibits the normal luteal regression, is a marker of trophoblast cell function early in gestation (Helmer *et al.* 1987, Roberts *et al.* 1992). IFN- τ mRNA expression is first seen around the time of blastocoel formation (Hernandez-Ledezma *et al.* 1992), and continues until just prior to attachment around days 25–28 of gestation, with the greatest expression during days 16–18 (Roberts *et al.* 1992). In the present study, no differences in IFN- τ mRNA expression were detected among day 17 AI, IVF, and SCNT embryos. These results indicate that mononucleate trophoblast cells in the SCNT embryos function normally with respect to expression of IFN- τ , thus aberrant maternal recognition of pregnancy may not account for the increased early pregnancy loss in SCNT embryos. In contrast, Stojkovic

et al. (1999) reported that in cultures, *in vivo*, IVF and SCNT embryos produce IFN- τ in a linear manner from days 11 to 15. However, after day 15, IFN- τ production from cloned embryos levels off, whereas *in vivo* and *in vitro* derived embryos continue to produce IFN- τ through day 23 (Stojkovic *et al.* 1999). In their experiments, embryos were cultured *in vitro* for the entire 23 days and did not elongate after day 9, as seen *in vivo*. In the present study, a more physiological model was employed in which embryos were transferred into recipient cows and allowed to develop *in vivo* until day 17, resulting in appropriate elongation. It has been reported that female bovine blastocysts produced twice as much IFN- τ as do male blastocysts (Larson *et al.* 2001). In the present study, the SCNT embryos had a male genotype, since the donor cells were derived from a day 60 male fetus. As we did not determine the sex of the AI and IVF embryos, it is not possible to determine if IFN- τ mRNA abundance results were effected by the sex of the embryos. Other researchers have reported that by day 14 the differences in IFN- τ due to sex are no longer apparent (Kimura *et al.* 2004) suggesting in the present study IFN- τ mRNA levels at day 17 are not different for the male and female embryos.

The expression of a pregnancy-associated glycoprotein isoform (PAG-9) mRNA was used as a marker of TGC function in day 17 embryos. Several isoforms of PAGs have been identified and they exhibit unique temporal and spatial expression patterns during pregnancy (Green *et al.* 2000). Of the 21 different isoforms, PAG-9 is expressed only by TGC as early as day 25 of gestation (Green *et al.* 2000) and appears to be the predominant PAG expressed at this time (Patel *et al.* 2004a). In this study, expression of PAG-9 mRNA was detected in day 17 AI embryos, but was undetectable in SCNT embryos. This concurs with reports that expression of PAG-1 and PAG-9 is lower in day 30 placental tissues of SCNT embryos compared to AI embryos (Patel *et al.* 2004b). The present study suggests that, in day 17 SCNT embryos, either no TGC are present or the TGC that are present are not functioning normally in terms to PAG-9 production. The specific role of PAGs in gestation remains unknown, however, some studies suggest that it may play a role in maternal immunosuppression during pregnancy (Dosogne *et al.* 2000). Evaluation of the inability of SCNT conceptuses to suppress the maternal immune system, allowing for normal maternal/fetal interaction awaits further examination.

To determine if altered expression of these genes are sustained as development continues, we chose to analyze cotyledonary tissue at day 40 of gestation, during the early phase of placentome formation. Abundance of Mash2 mRNA remained elevated in the SCNT group relative to both the IVF and AI groups. This sustained elevation suggests that either epigenetic regulation of Mash2 was not correctly reprogrammed in the donor cell or genes that regulate Mash2 were

altered due to SCNT. Incomplete epigenetic reprogramming has been proposed to cause altered X chromosome inactivation and bi-allelic expression of the maternally expression X-linked monoamine oxidase type A gene in placental tissue of deceased cloned cattle (Xue *et al.* 2002). In the present study, bi-allelic expression of Mash2 observed in all day 17 samples supports data suggesting that bovine Mash2 is expressed by both alleles prior to implantation and maternally expressed after implantation (Arnold *et al.* 2006). At day 40, the paternal allele of Mash2 appears to be silenced, even though low levels of paternal expression were detected in a few samples, regardless of group. These data indicate that altered Mash2 expression may be due to genes that regulate Mash2 and not to direct alteration of the imprinting status of Mash2.

By day 40, the abundance of Hand1 has become higher in the SCNT group relative to the AI and IVF controls. If Mash2 and Hand1 function as opposing factors (Cross *et al.* 1995), the higher Hand1 expression could be a response to the elevated Mash2 mRNA in SCNT trophoblast. Similar to the data that overexpressing Hand1 Rcho-1 cells that co-expressed Mash2 had inhibited giant cell development (Cross *et al.* 1995), the SCNT trophoblast cells may not be able to stimulate TGC differentiation.

With evidence for altered expression of Mash2 and Hand1 mRNA in SCNT embryos and cotyledonary tissue, the next question to ask is how may these differences affect TGC densities and function. Klisch *et al.* (1999) reported that bovine TGC could be identified; by the number of nuclei, by the shape of the cell, and by the presence of PAS positive granules within the cells. These criteria were utilized in the present study to determine differences in TGC densities. The cotyledonary tissue from SCNT embryos contained fewer TGC than the AI and IVF samples. The values for the AI samples are similar to those reported by other researchers (Wooding 1982, 1983). Other investigators have reported no differences between TGC cell numbers for IVF and SCNT day 30 samples (Hoffert *et al.* 2005). These authors nonetheless reported higher than expected numbers of TGC for both groups (26 and 25.7% for SCNT and IVF samples respectively). These differences may be due to the type of tissue analyzed or the embryo culturing protocols. In the present study, day 40 of gestation was chosen so that the cotyledonary tissue would be more developed and could be distinguished from the intercotyledonary tissue. In addition, only the fetal placental tissue was analyzed. Even though the day 40 placentome allowed for the identification of the placentomes, the interdigitation at this time is rudimentary, allowing for fetal and maternal tissues to be separated. Additionally, the present study employed *in vitro* culture of the IVF and SCNT groups through to the blastocyst stage (day 8), whereas Hoffert *et al.* (2005) cultured embryos to the blastocyst stage in ligated

sheep oviducts to mimic *in vivo* conditions. To address any effects due to *in vitro* culturing (for review, see Farin *et al.* 2004) in the present study, *in vivo* produced embryos were also analyzed. The densities of TGC were similar between the AI and IVF groups, indicating that the reduced number of TGC in the SCNT samples were due primarily to the cloning procedure.

To further analyze TGC densities and determine whether TGC from SCNT cotyledons were functioning properly, a MAB that recognizes PAG was employed (Lee *et al.* 1986). Similar to the density of TGC, SCNT cotyledonary tissue had fewer positive cells than its AI and IVF counterparts. Interestingly, the IVF group had fewer positive cells than did the AI. In contrast to these findings, Ravelich *et al.* (2004a), reported greater numbers of fetal, maternal, and binucleate cells in NT placentomes at days 50, 100, and 150, compared to AI/IVF controls. These differences may be due to the days examined and the proteins detected. In the present study, we utilized a MAB that specifically recognizes PAGs from binucleate/giant cells in the cotyledonary region (Lee *et al.* 1986), whereas the latter study utilized immunostaining for bovine placental lactogen (Ravelich *et al.* 2004a). The data provided in the present study support the *in situ* hybridization data that PAG mRNA is reduced in cotyledonary tissue of day 60 SCNT placentas compared to AI controls (Hashizume *et al.* 2002). It further suggests that TGC from SCNT cotyledonary tissue are able to function similar to AI and IVF TGC, with respect to PAG production. However, with the reduced number of cells to produce PAG, overall SCNT placental function may be compromised. As stated above, the function of PAGs is not fully understood. However, Chavatte-Palmer *et al.* (2006) reported lower serum PAG concentrations in recipients which experienced early pregnancy loss (between days 35 and 90 of gestation) of SCNT fetuses. Incidentally, recipients who carried SCNT pregnancies to term had higher concentrations of PAG early in gestation compared to IVF controls (Chavatte-Palmer *et al.* 2006). These results may explain the differences in the present study with those previously reported using placental lactogen as a marker (Ravelich *et al.* 2004b). It must be kept in mind that 75% SCNT embryos did not survive to day 40 in the present investigation. It is possible that the embryos available for study after this time survived because sufficient numbers of TGC were present to allow for implantation and cotyledon development to occur.

In summary, we provide new information to demonstrate that genes critical for trophoblast proliferation (Mash2) and differentiation (Hand1) are aberrantly expressed in embryos derived from SCNT, which have recognizable placental abnormalities. In addition, trophoblast giant cell development is reduced in SCNT cotyledonary tissues. We believe that a plausible mechanism for altered trophoblast development in SCNT bovine embryos is the incomplete epigenetic

reprogramming of the donor cells. This altered reprogramming (either directly or indirectly) may cause the Mash2 gene to be overexpressed and the expression of Hand1 to be delayed early in development and overexpressed as pregnancy progresses. This series of events mimics those seen in mouse models where no Hand1 early in development leads to embryonic death due to reduced giant cell formation. We further report that placental tissues from SCNT fetuses had fewer TGC. Fewer TGC cause a reduction in overall PAG production, which could in itself cause an inability of the fetal unit to immunosuppress the maternal environment, leading to pregnancy loss commonly associated with nuclear transfer. By understanding the underlying molecular events involved in bovine trophoblast development, we can gain insight into the regulatory mechanisms involved in successful placentation and how these events may be manipulated to improve assisted reproductive techniques, such as SCNT.

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