Cytokine Gene Expression in the Maternal-Fetal Interface in Somatic Cell Nuclear Transfer Pregnancies in Small Ruminants

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Short title: Cytokines in Cloned Sheep and Goat Pregnancies

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Abstract

The present retrospective study investigates pregnancy rates, incidence of pregnancy losses and large offspring syndrome (LOS), and immune-related gene expression of sheep and goat somatic cell nuclear transfer (SCNT) pregnancies. We hypothesized that significantly higher pregnancy losses observed in sheep SCNT pregnancies compared to goats are due to the increased amounts of T-helper 1 cytokines and pro-inflammatory mediators at the maternal-fetal interface. Sheep and goat SCNT pregnancies were generated using the same procedure. Control pregnancies were established by natural breeding. Although SCNT pregnancy rates at 45 days were similar in both species, pregnancy losses between 45 and 60 days and incidence of LOS were significantly increased in sheep compared with goats. At term, the expression of pro-inflammatory genes in sheep SCNT placentas was increased while the one of goat SCNT was similar to the control animals. Among the genes that had altered expression in sheep SCNT placentas are *CTLA4, IL2RA, CD28*, *IFNG, IL6, IL10, TGFB1, TNF, IL1A and CXCL8*. MHC-I protein expression was greater in sheep and goat SCNT placentas at term compared with control pregnancies. An unfavorable immune environment is present at the maternal-fetal interface in sheep SCNT pregnancies.

1. Introduction

In sheep and cattle, pregnancies generated by somatic cell nuclear transfer (SCNT) are at increased risk of early pregnancy loss, late term pregnancy complications

(Campbell *et al.*, 1996; Schnieke *et al.*, 1997; Wells *et al.*, 1997; Wilmut *et al.*, 1997; Edwards *et al.*, 2003; Fasouliotis and Schenker, 2003; Shevell *et al.*, 2005; Loi *et al.*, 2006) and large offspring syndrome (LOS) (Behboodi *et al.*, 1995; Young *et al.*, 1998; Wilmut *et al.*, 2002; Constant *et al.*, 2006). For instance, approximately 50% of SCNT generated full-term calves and lambs are diagnosed with LOS (Constant *et al.*, 2006). In addition to fetal abnormalities, calves with LOS also present placental anomalies, fewer and enlarged placentomes and reduced placental vascularization (Hill *et al.*, 2000; Bertolini and Anderson, 2002; Chavatte-Palmer *et al.*, 2002; Constant *et al.*, 2006). In goats, SCNT outcomes have been variable with studies showing pregnancy loss after day 60 of gestation(Baguisi *et al.*, 1999; Keefer *et al.*, 2001, 2002; Reggio *et al.*, 2001); while others report pregnancy losses of approximately 100% (Zhu *et al.*, 2009; Zhou *et al.*, 2013).. The increased pregnancy loss observed in SCNT pregnancies may be due, at least in part, to a deficient cross talk between the mother and the fetus. Abnormal trophoblast gene expression patterns in SCNT pregnancies have been observed in various species (Bauersachs *et al.*, 2009; Mansouri-Attia *et al.*, 2009; Rodríguez-Alvarez *et al.*, 2010a; Isom *et al.*, 2013). Expression of genes related to immune responses, metabolism, oxidative phosphorylation, cellular response to hypoxia and angiogenesis is misregulated in bovine SCNT pregnancies (Mansouri-Attia *et al.*, 2009).

A shift from a T-helper 1 (Th1) to a T-helper 2 (Th2) response is an important factor in the maintenance of pregnancy in humans and mice. In the uterus of a non-pregnant woman there is a homeostasis between Th1 and Th2 activity (Sargent *et al.*, 2006). In normal pregnancies, this balance is shifted toward a Th2 type response because of the presence of progesterone and placental cytokines (Piccinni *et al.*, 2000). However,

70 an extended Th1 response has been associated with recurrent miscarriages in humans
71 (Jenkins *et al.*, 2000; Lim *et al.*, 2000). In abortion-susceptible mouse models, fetal loss
72 has been associated with the expression of Th1 cytokines and deficient expression of Th2
73 cytokines (Chaouat *et al.*, 1990, 1995). The production of Th2 cytokines, mainly IL4,
74 IL5, IL6, IL10 and IL13, promotes growth of trophoblast cells and may help maintain
75 pregnancy (Lin *et al.*, 1993; Wegmann *et al.*, 1993). Conversely, Th1 cytokines such as
76 IFNG, TNF and IL2 contribute to placental toxicity and damage, directly or indirectly
77 through the activation of other immune cells (Arck *et al.*, 1999; Lim *et al.*, 2000).
78 Recently, it has been reported that a shift towards a Th2 cytokine response is associated
79 with normal pregnancies in cattle (Oliveira *et al.*, 2013).

80 In this retrospective study we determined the pregnancy rates and the incidence of
81 pregnancy losses and LOS of sheep and goat SCNT pregnancies. We also investigated
82 the immune-related gene expression profile of placentas originated from sheep and goat
83 SCNT and from naturally conceived pregnancies. Our hypothesis is that in SCNT-
84 generated pregnancies, pregnancy losses between 45 days and term in sheep are
85 significantly higher than in goats due to the increased amounts of Th1 cytokines and pro-
86 inflammatory mediators at the fetal-maternal interface, which contribute to placental
87 dysfunction and pregnancy loss.

88

89 *2. Material and Methods*

90 *2.1. Somatic Cell Nuclear Transfer*

Sheep and goat SCNT pregnancies were generated as described by (Hall *et al.*, 2012). Passage 2-5 fibroblast cells were grown to 90-100% confluence and used as nuclear donor cells after 24 hours of serum starvation (0.5% FBS, Hyclone Laboratories, Logan, UT, USA). Cumulus-oocyte complexes were recovered from ovaries using slicing and aspiration techniques. The quality of collected oocytes was assessed based on morphology. All good and fair quality oocytes were cultured in maturation medium as described elsewhere (Reggio *et al.*, 2001). After 22 to 24 hours of culture, cumulus cells were removed from matured oocytes and oocytes with a first polar body were used as recipient cytoplasts. The first polar body and metaphase plate were removed, and subsequently single donor cells were transferred to the perivitelline space of recipient cytoplasts. Fusions of somatic cells with oocyte cytoplasm were performed in sorbitol fusion medium (0.28 M sorbitol, 100 μM calcium acetate, 0.5mM magnesium acetate and 1 mg/ml BSA) by a single DC electric pulse of 1.75 kV/cm for 15 microseconds. Fusion of the donor cell with oocyte cytoplasm was evaluated by microscopy 30 minutes after the pulse. Fused embryos were activated between 27 and 29 hours after the onset of maturation by exposure to 5 μM ionomycin (Sigma-Aldrich, St. Louis, MO, USA) for 5 minutes followed by a 4-hour incubation in 2 mM DMAP (Sigma-Aldrich, St. Louis, MO, USA) and 10 μg/ml cycloheximide (Sigma-Aldrich, St. Louis, MO, USA). Following activation, goat embryos were cultured in G1 medium (Vitrolife, Goteborg, Sweden) and sheep embryos were cultured in either G1 or SOF (Walker *et al.*, 1996) media for 12 hours. Since no difference was observed in the pregnancy and LOS rates, and gene expression in sheep SCNT pregnancies using two different culture media the data were combined.

114 *2.2. Establishment of Pregnancies and Sample Collection*

115 The use of animals for this study was approved by the Institutional Animal Care
116 and Use Committee at Utah State University. Eighty-two domestic sheep (*Ovis aries*) and
117 37 domestic goats (*Capra aegagrus hircus*) were used as recipients for embryo transfers.
118 All animals were housed in an open sided barn with free access to food and water.
119 Experiments were conducted simultaneously in both species. Somatic cell nuclear
120 transfer pregnancies were established by surgically transferring 16 ± 3 embryos into the
121 oviduct of recipients synchronized to show estrus within 12 hours of SCNT.
122 Confirmation of pregnancy was determined by ultrasonography on days 45 and 60 of
123 gestation.

124 A subset of the SCNT pregnancies was used for placental gene expression
125 analysis (sheep: n=6; goat: n=8). Since the main objective of the present study is to
126 identify the immune-related genes that are altered in SCNT compared to normal
127 pregnancies, control pregnancies (sheep: n=6; goat: n=8) were established by natural
128 breeding. Parturition in animals that did not deliver naturally by 152 ± 1 days of gestation
129 was pharmacologically induced by intramuscular administration of dexamethasone (20
130 mg for sheep and 12 mg for goats) and prostaglandin F2α (10 mg for sheep and 15 mg for
131 goats).

132 Immediately after vaginal delivery, intercotyledonary and cotyledonary chorionic
133 samples were collected separately, snap frozen in liquid nitrogen and stored at -80°C. For
134 immunohistochemistry, placental samples collected from the ipsilateral horn to the
135 pregnancy were frozen in a Tissue-Tek optimal cutting temperature (Sakura,
136 Flemingweg, The Netherlands) compound. Eight μm thick sections acquired using a

cryostat microtome, were placed on pre-cleaned Superfrost Plus microscope slides, fixed in ice-cold acetone for 5 minutes, air-dried and then frozen at -80°C for long term storage.

2.3. RNA Extraction and Gene Expression Analysis

Total RNA was extracted from snap frozen tissues using the TRizol Plus Purification System (Life Technologies, Grand Island, NY, USA). Concentration of total RNA was determined with a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and RNA integrity was determined using a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

Five µg of RNA were reverse transcribed using SuperScript™ VILO cDNA synthesis kit and master mix (Life Technologies, Grand Island, NY, USA) according to manufacturer's instructions. A pre-amplification step was performed prior to the Fluidigm high throughput qPCR. One µl of each primer pair of interest (100 µM each) was pooled and made to a final volume of 100 µl of 1x TE buffer, pH 8.0. In order to make a 5 µl pre-amplification reaction, 1.25 µl of each sample was added to 2.5 µl of Preamp Master Mix (Life Technologies, Grand Island, NY, USA), and 1.25 µl of pooled primer mix. The final concentration of each primer was 50 nM. cDNA was amplified for 14 cycles under the following conditions: 95°C for 15 seconds, and 60°C for 4 minutes. Unincorporated primers were removed by treating amplified cDNA with Exonuclease 4U/ µl (New England Biolabs, Whitby, Ontario, Canada) for 30 minutes at 37°C and 15 minutes at 80°C. This mixture was then diluted 1:5 with RNAse and DNAase free water.

159 Eva Green™ high-throughput nanoliter volume microfluidic chip quantitative
160 RT-PCR (48.48 Dynamic Array; Fluidigm Corporation, South San Francisco, CA, USA)
161 was used to determine the level of gene expression for the following genes: IL1A, IL2,
162 IL4, IL5, IL6, CXCL8, IL10, IL12B, IL13, IL15, IL17A, IL18, IL23A, IFNG, TNF,
163 TGFB1, CSF2, IL2RA, CD28, CTLA4, GATA3, TBX21, GNLY, MHCI, IFNA2. Primer
164 details are shown in Table 1. Two primer sets for glyceraldehyde- 3-phosphate-
165 dehydrogenase (GAPDH) and β-actin (ACTB) were used as housekeeping genes.
166 Standard PCR reactions were performed to confirm the specificity of each primer set.
167 Quantitative RT-PCR reactions were performed following the standard Fluidigm protocol
168 (Spurgeon *et al.*, 2008). A 48.48 Dynamic Array chip (Fluidigm Corporation, South San
169 Francisco, CA, USA) was first primed with Krytox in the IFC controller (Fluidigm
170 Corporation, South San Francisco, CA, USA). Then, 5 µl sample mixtures containing 2.5
171 µl of 2x TaqMan Gene Expression Master Mix (Life Technologies, Grand Island, NY,
172 USA), 0.25 µl of DNA sample loading reagent (Fluidigm Corporation, South San
173 Francisco, CA, USA), 0.25 µl of EvaGreen DNA binding dye (Biotium, Hayward, CA,
174 USA) and 2 µl of pre-amplified cDNA sample were pipetted into the sample inlets of the
175 chip. Five µl assay mix containing 2.5 µl of 2x assay loading reagent (Fluidigm
176 Corporation, South San Francisco, CA, USA), 0.25 µl of 1x TE buffer and 2.25 µl of
177 primer pairs (20 µM) were pipetted into the assay inlets and chip was loaded in the IFC
178 controller (Fluidigm Corporation, South San Francisco, CA, USA). Quantitative RT-PCR
179 was performed using the Biomark Real-Time PCR System (Fluidigm Corporation, South
180 San Francisco, CA, USA) under the following conditions: 10 minutes at 95°C followed
181 by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

182 Data were analyzed using Fluidigm Real-Time PCR Analysis software version
183 3.02 (Fluidigm Corporation, South San Francisco, CA, USA) to yield relative
184 quantitation values calibrated to control animals. Analysis of variance was used to
185 determine that amplification of the housekeeping genes, GAPDH and ACTB, was not
186 statistically different across groups (P = 0.48). Relative gene expression data was
187 analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using the average of the
188 housekeeping genes *GAPDH and ACTB* for normalization. The values presented here
189 reflect the fold change of gene expression in the SCNT groups in sheep and goats
190 compared with the control groups of the respective species. The fold change in gene
191 expression was only considered biologically significant if above 2.

192

193 *2.4. Immunohistochemistry*

194 Slides containing frozen sections were allowed to thaw at room temperature and
195 then rehydrated in 2 changes of PBS for 10 minutes. Sections were treated with 0.3%
196 hydrogen peroxide in PBS for 10 minutes to block endogenous peroxidase activity. All
197 incubations were done at room temperature in a humidity chamber and slides were
198 washed in three changes of PBS between incubations except for when the blocking
199 solution and primary antibody incubation treatments were used. Nonspecific binding sites
200 were blocked with PBS containing 1% bovine serum albumin (BSA) and 2% normal goat
201 serum for 20 minutes. Immediately after treatment with blocking solution, sections were
202 incubated for one hour with anti-H58 monoclonal primary antibody (Washington State
203 University Monoclonal Antibody Center, Pullman, WA, USA). This antibody reacts

204 strongly with ovine and caprine MHC-I proteins (Davis *et al.*, 1987). Sections were then
205 treated with 1 ml of 7.5 μg/ml of biotinylated goat anti-mouse IgG secondary antibody
206 (Vector Laboratories, Burlingame, CA, USA) for 20 minutes followed by streptavidin
207 peroxidase incubation for 20 minutes. Slides were incubated with 3-amino-9-
208 ethylcarbazole (AEC) kit (Life Technologies, Grand Island, NY, USA) for 5 minutes and
209 excess AEC was removed by washing in distilled water. Sections were counterstained
210 with haematoxylin for 1 minute and the excess was removed by washing in distilled
211 water. Slides were mounted using the water-soluble Fluoromount-G mounting medium.
212 Stained sections were analyzed using a Zeiss Axio Observer microscope (Zeiss,
213 Gottingen, Germany) with a 10x objective. Digital images were acquired using
214 AxioVision software (Zeiss, Gottingen, Germany) and a high-resolution AxioCam HRC
215 digital camera. The MHC-I^+ protein expression levels of trophoblast cells was assessed by
216 the area percent of the total trophoblast area that was occupied by these cells using the
217 AxioVision software (Zeiss, Gottingen, Germany).

218

219 *2.5. Statistical Analysis*

220 Analyses of gene expression employed one-way ANOVA (analysis of variance)
221 models using the MIXED procedure of SAS (SAS for Windows, version 9.3, SAS
222 Institute Inc., Cary, NC, USA) with treatment as the sole fixed effect and cell type and
223 embryo culture medium as covariables. Significant differences between treatments were
224 determined by t-test with the pdiff option and Tukey's adjustment. Treatment effects on
225 pregnancy rates, pregnancy losses and incidence of LOS were examined by chi-squared

analysis using the FREQ procedure of SAS (SAS for Windows, version 9.3, SAS Institute Inc., Cary, NC, USA). Effects were considered to be significant when the *P* value was equal or below 0.05.

3. Results

3.1. Pregnancy Rates and Pregnancy Loss

As depicted in Table 2, 82 SCNT embryo transfers were performed in sheep and 37 in goats. Pregnancy rates at 45 days after SCNT were similar ($P = 0.38$) between the two species (32.9% for sheep and 32.4% for goats). Pregnancy rates declined ($P = 0.042$) at 60 days in sheep compared to goats (19.5% versus 32.4%, respectively). In sheep, pregnancy rates at term did not differ from day 60 suggesting that the critical period for loss of SCNT pregnancies in sheep occur between days 45 and 60 of gestation. A significant difference ($P < 0.001$) was observed between sheep and goat in pregnancy losses between 45 days and term with 11 of 27 pregnancies (40.7%) being lost in sheep while none of the 12 pregnancies were lost in goats (Table 2).

For pregnancies generated by natural breeding, day 45 pregnancy rates were similar ($P = 0.94$) with 82% (18/22) and 81% (17/21) for sheep and goats, respectively and losses between gestation day 45 and term were 5.6% (1/18) for sheep and 5.9% (1/17) for goats ($P = 0.97$).

3.2. Incidence of Large Offspring Syndrome

247 Large offspring syndrome was characterized by increased birth weight combined
248 with placental and/or fetal anomalies such as: enlarged organs and umbilical cord,
249 hydrops of the fetus, lethargy, skeletal and cranial malformations and abdominal wall
250 defects. Incidence of LOS was greater (P = 0.03) in sheep SCNT pregnancies than in
251 goats SCNT pregnancies (31.3% versus 0%, respectively; Table 2).

252

253 3.3. Expression of Genes Related to Immune Function in the Placenta

254 Our data indicate that the expression patterns of genes related to immune function
255 are aberrant in sheep placental samples from SCNT-generated pregnancies compared
256 with pregnancies established by natural breeding, and goat SCNT pregnancies.

257 The intercotyledonary region of sheep SCNT placentas showed upregulation ($P <$
258 0.05) of immune-related genes such as *CTLA4, IL2RA, CD28*, *IL6, TGFB1, IL1A and*
259 *CXCL8,* while goat SCNT placentas showed no significant change in expression of such
260 genes relative to pregnancies established by natural breeding. Sheep SCNT
261 intercotyledonary placentas also expressed greater levels of Th1 cytokines such as TNF
262 and IFNG. Upon comparing goat and sheep SCNT intercotyledonary placentas, sheep
263 had greater ($P < 0.04$) mRNA expression of the above-mentioned genes (Fig. 1).

264 As shown in Figure 2, the cotyledonary region followed a similar pattern of gene
265 expression as the intercotyledonary region. Sheep SCNT placentas had greater ($P < 0.05$)
266 mRNA expression levels of *CD28, IL10, IL1A, CXCL8, TGFB1* and *TNF* compared with
267 sheep control placentas and to goat SCNT placentas. Whereas, CSF2 expression was

268 greater in SCNT goats compared with SCNT sheep (P = 0.035) and with control goat
269 placentas (P = 0.022).

270 In the intercotyledonary region of the placenta, the level of expression of *CTLA4,*
271 *IL2RA, CD28*, *IFNG, IL6, TGFB1, TNF, IL1A and CXCL8* was similar (P > 0.2) between
272 sheep and goat control pregnancies (Fig. 3A). Comparably, expression levels of *CD28,*
273 *CSF2, IL10, IL1A, CXCL8, TGFB1 and TNF* were similar (P > 0.21) in the cotyledonary
274 region of sheep and goat control placentas (Fig. 3B).

275

276 3.4. MHC-I Expression in the Trophoblast

277 The expression levels of MHC-I were examined by quantitative RT-PCR and
278 immunohistochemistry in the trophoblast cells of sheep and goat pregnancies established
279 by SCNT and natural breeding. The gene expression levels of MHC-I was greater (P <
280 0.05) in the intercotyledonary region of the placenta of sheep and goat SCNT pregnancies
281 than in their respective control groups. Major histocompatibility complex class I gene
282 expression did not differ between sheep and goat SCNT pregnancies. Gene expression
283 findings were in agreement with protein expression (Fig. 4; data not shown).

284

285 4. Discussion

286 This study is the first of its kind to demonstrate a direct comparison between
287 successful and abortion prone SCNT pregnancies in which pregnancy, embryonic loss
288 and LOS rates were compared in sheep and goat SCNT pregnancies established under the
289 same conditions. The major finding of this study was the identification of a pro-
290 inflammatory cytokine pattern at the maternal-fetal interface in abortion-prone

291 pregnancies (sheep SCNT pregnancies). The data also showed that these pregnancies
292 generate a high percentage of LOS fetuses.

293 Assisted reproductive technologies are used for faster dissemination of desirable
294 traits in production herds (Mapletoft and Hasler, 2005; Polejaeva *et al.*, 2013). Tracking
295 by the International Embryo Transfer Society indicates that nearly 374,000 *in vitro*-
296 produced bovine embryos were transferred worldwide in 2011, a 10% increase from 2010
297 ('International Embryo Transfer Society (IETS)'). In humans, pregnancies generated by
298 ART account for 1-3% of births in developed countries. The increased risk of
299 embryonic/fetal loss and late term complications in pregnancies generated by ART has
300 limited a broader use of the technology (Edwards *et al.*, 2003; Fasouliotis and Schenker,
301 2003; Shevell *et al.*, 2005). The mechanisms causing these reproductive problems are still
302 not fully understood.

303 Similarly to our findings in this study, multiple reports have shown high
304 pregnancy losses following SCNT in sheep ranging from 34 to 62% between day 60 of
305 gestation and term (Campbell *et al.*, 1996; Schnieke *et al.*, 1997; Wells *et al.*, 1997;
306 Wilmut *et al.*, 1997; Loi *et al.*, 2006). Up to 40% of full-term lambs exhibit LOS (Wells
307 *et al.*, 1997; Young *et al.*, 1998, 2003; Fletcher *et al.*, 2007) and perinatal mortality can
308 be as high as 100% (Schnieke *et al.*, 1997; Wells *et al.*, 1997; Loi *et al.*, 2006). In goats
309 the outcome is different where birth weights of SCNT-derived goats are typically within
310 the normal range (Reggio *et al.*, 2001; Keefer *et al.*, 2002; Lan *et al.*, 2006; Amiri Yekta
311 *et al.*, 2013), except for one report of a LOS phenotype in a male goat (Chen *et al.*, 2002).
312 Pregnancy losses reported in goat SCNT vary substantially between different research
313 groups. Several groups have shown that when goat SCNT pregnancies reach 30 or 60

314 days they will typically go to term with no perinatal mortality (Baguisi *et al.*, 1999;
315 Keefer *et al.*, 2001, 2002; Reggio *et al.*, 2001); whereas, other studies describe pregnancy
316 losses of 100% (Zhu *et al.*, 2009; Zhou *et al.*, 2013). Since most of the reports are using
317 transgenic fibroblasts, the type of transgene, the oocyte activation method, the length of
318 cell culture and the type of selection pressure applied to the fibroblasts prior to SCNT
319 likely contributes to differences in pregnancy rates and losses. The time of pregnancy
320 detection could also contribute to outcome differences. Ultrasonography at 30-35 days of
321 gestation often fails to detect fetal heartbeats in sheep and goats (personal observation)
322 and therefore, trophoblastic vesicles (in the absence of an embryo proper) are often
323 mistaken as pregnancies (Baguisi *et al.*, 1999; Zhou *et al.*, 2013). This would result in
324 false positive pregnancies leading to superficially higher pregnancy losses. To avoid
325 potential bias, all pregnancies in this study were confirmed positive only if a fetal
326 heartbeat could be detected.

327 Although the etiology of LOS and placental insufficiency has not been fully
328 elucidated abnormal gene expression at the maternal-fetal interface has been described by
329 several research groups. (Mansouri-Attia *et al.*, 2009) observed abnormal transcriptome
330 profiles in endometrial samples collected from bovine pregnancies generated by SCNT
331 compared to those generated by artificial insemination. Most of the genes that are
332 abnormally expressed in these pregnancies are related to immune responses, metabolism,
333 oxidative phosphorylation, cellular response to hypoxia and angiogenesis (Mansouri-
334 Attia *et al.*, 2009). Consistent with altered expression of hypoxia- and angiogenesis-
335 related genes, impaired vascular development is seen in both sheep and bovine concepti
336 generated by SCNT (Hill *et al.*, 2000, 2002; De Sousa *et al.*, 2001; Palmieri *et al.*, 2007).

337 Trophoblast gene expression is also abnormal in SCNT and *in vitro* fertilization
338 pregnancies in various species (Bauersachs *et al.*, 2009; Mansouri-Attia *et al.*, 2009;
339 Rodríguez-Alvarez *et al.*, 2010a, b; Isom *et al.*, 2013).

340 Our data show that the expression profile of immune-related genes in sheep
341 SCNT placentas at term is aberrant. These pregnancies showed significant upregulation
342 of pro-inflammatory genes whereas goat SCNT pregnancies did not show a change in
343 expression of the same genes relative to natural breeding. This suggests that, at least in
344 part, the low survival rate of sheep SCNT embryos and fetuses is caused by a lack of
345 immune-mediated mechanisms that protect the fetus from the maternal immune system.
346 Immunological rejection of SCNT fetuses could be a consequence of a breakdown of
347 mechanisms that prevent the maternal immune system from becoming activated by
348 antigens expressed by the developing fetus.

349 We have determined that upregulation of genes *IL2RA, IFNG, IL6, TNF, IL1A*
350 *and CXCL8* in the placenta of SCNT sheep pregnancies is significantly more pronounced
351 than in the SCNT goat pregnancies. The increased expression of these proteins has been
352 associated with miscarriages in humans (Shaarawy and Nagui, 1997; Wang *et al.*, 2010;
353 Galazios *et al.*, 2011; Jin *et al.*, 2011a; Prins *et al.*, 2012). The balance between trophic
354 and toxic cytokines seems to determine the fate of a developing conceptus. It has been
355 proposed that pregnancy depends on a bias towards Th2 type immune responses rather
356 than Th1. A predominant Th2 cytokine profile favors pregnancy whereas a Th1 biased
357 response has been associated with pregnancy loss in humans and mice (Chaouat *et al.*,
358 1990, 1995; Jenkins *et al.*, 2000; Lim *et al.*, 2000). A Th1 biased response causes an
359 inflammatory reaction, with the increase in IFNG and TNF likely to contribute to

placental toxicity and subsequent pregnancy failure in humans (Raghupathy, 1997) and mouse models (Tangri and Raghupathy, 1993). The excess of these pro-inflammatory cytokines also skews the adaptive immune response towards cytotoxicity and away from generation of T regulatory cells (Trowsdale and Betz, 2006; Moldenhauer *et al.*, 2009; Robertson *et al.*, 2009; Shima *et al.*, 2010). IL1A, TNF and IFNG have been shown to be elevated in serum samples of patients experiencing recurrent miscarriages. IL2R has a role in cell-mediated inflammation and in promoting Th1 activation and has been shown to be upregulated in decidual chorionic tissue in cases of recurrent miscarriages during the first trimester (Giannubilo *et al.*, 2012). Additionally, IL6 and CXCL8 were elevated in serum samples of women who had second trimester miscarriages (Galazios *et al.*, 2011). Although a Th2 cytokine, IL6 levels are often elevated in cytokine profiles characteristic of infertility, recurrent pregnancy loss and complications (Prins *et al.*, 2012).

Here we have shown that TGFB1 expression follows the same pattern as the other cytokines with sheep SCNT placentas having greater expression than the other groups. TGFB1 has multiple functions within and outside the immune system. It promotes the generation of T regulatory cells and is involved in cell proliferation, differentiation, angiogenesis and tissue remodeling. The role of TGFB1 during pregnancy is still controversial. TGFB1 has a role in trophoblast invasion and its expression is upregulated in preeclamptic placentas and its inhibition restores the invasive capacity of trophoblast cells (Caniggia *et al.*, 1999); whereas, Giannubilo *et al*. (2012) reported that TGFB1 expression is downregulated in recurrent miscarriages.

382 Although IL10 is a Th2 cytokine and it has been associated with trophoblast
383 growth and maintenance of pregnancy (Lin *et al.*, 1993; Wegmann *et al.*, 1993), we have
384 observed that this cytokine is upregulated in the intercotyledonary regions of sheep
385 SCNT placentas. Similarly, Rosbottom *et al.* (2011) reported that IL10 was upregulated
386 in the placenta of cattle infected with *Neospora caninum.* This upregulation could be a
387 compensatory effect to preserve integrity and homeostasis of the endometrium epithelium
388 during inflammation (reviewed by Ouyang *et al.*, 2011)

389 We observed that the cotyledonary region of term goat SCNT placentas has
390 increased levels of CSF2 compared to the control groups and sheep SCNT. In cattle
391 (Loureiro *et al.*, 2009; Denicol *et al.*, 2014), humans (Ziebe *et al.*, 2013), mice
392 (Robertson *et al.*, 2001; Sjöblom *et al.*, 2005) and pigs (Lee *et al.*, 2013) the addition of
393 CSF2 to embryo culture media has been shown to promote blastocyst development and to
394 increase implantation success. CSF2 also has been shown to improve embryonic cell
395 survival, inhibit apoptosis and facilitate glucose uptake (Robertson *et al.*, 2001; Chin *et*
396 *al.*, 2009). Although the function of CSF2 has not yet been completely elucidated in term
397 pregnancies, it appears to have a role in the development and maintenance of a fully
398 functional placenta in goat SCNT pregnancies possibly due to an inhibition of trophoblast
399 cell apoptosis.

400 Furthermore, we compared the upregulation of CD28 and CTLA4 in sheep with
401 goat SCNT and control pregnancies. CD28 and CTLA4 are co-stimulatory receptors
402 involved in regulating immune responses. CD28 expression has been correlated with Th1
403 cytokine response, while CTLA4 exerts an inhibitory effect on the immune system (Liu,
404 1997; Chambers, 2001). Even though the mechanisms underlying the regulation of the

405 maternal-fetal immune response by these factors are largely unknown, studies have
406 shown that the expression of CD28 is upregulated and CTLA4 downregulated in first
407 trimester decidual tissues of miscarriages (Jin *et al.*, 2011a, b), and that the CTLA4/CD28
408 ratios in miscarriage cases were observed to be lower than in normal pregnancies (Jin *et*
409 *al.*, 2009). Here we observed that the ratio of CTLA4/CD28 in goat SCNT pregnancies
410 (1.37) is significantly reduced compared with the ratio in sheep SCNT (0.75; data not
411 shown), while ratios in goat and sheep control pregnancies are similar (1.32 and 1.30,
412 respectively). .

413 To the best of our knowledge there is only one study examining MHC-I
414 expression by trophoblast cells in sheep where the MHC-I protein was not detected at any
415 time of the pregnancy (Gogolin-Ewens *et al.*, 1989). Trophoblast MHC-I expression in
416 cattle has been investigated in greater details. (Davies *et al.*, 2000) reported that its
417 expression is temporally and regionally regulated in the placenta and that trophoblast
418 cells downregulate MHC-I expression in the first trimester of pregnancy, which is most
419 likely a mechanism to protect the semiallogeneic conceptus from recognition by the
420 maternal immune system. In the intercotyledonary region a significant number of
421 trophoblast cells were positive for classical and non-classical MHC-I proteins from the
422 sixth month on of pregnancy (Davies *et al.*, 2000). In the cotyledonary villi, the area of
423 intimate contact between fetal cells and the maternal epithelium, trophoblast cells were
424 negative for MHC-I proteins throughout gestation (Davies *et al.*, 2000). The
425 downregulation of trophoblast MHC-I expression during the first trimester in cattle seems
426 to be essential to prevent a maternal immune response to fetal proteins. Cattle SCNT
427 derived pregnancies express abnormal amounts of classical MHC-I proteins on the

428 surface of trophoblast cells during the first trimester and this is associated with
429 infiltration of mainly $CD3^+$ T lymphocytes into the endometrium (Hill *et al.*, 2000).

430 This is the first study undertaken to investigate MHC-I expression in placentas of
431 SCNT pregnancies in sheep and goats. Our data suggest that MHC-I expression by
432 trophoblast cells is over 10 times greater in the intercotyledonary region of both sheep
433 and goat SCNT placentas compared with placentas originated from natural breeding (Fig.
434 3); whereas, gene expression of pro-inflammatory cytokines was upregulated only in the
435 sheep SCNT pregnancies. There are three possible explanations for this observation. The
436 most likely explanation is that trophoblast cells express MHC-I proteins on their surface
437 earlier in sheep SCNT pregnancies than in goat SCNT pregnancies, which could trigger a
438 more severe immune response leading to pregnancy loss and complications.

439 Since the primers used in this study could not differentiate mRNA encoding
440 classical and non-classical MHC-I, a second possibility is that MHC-I expression in goat
441 SCNT placentas is predominantly composed of non-classical MHC-I proteins while in
442 sheep it is predominantly composed of classical MHC-I proteins. There are two
443 subclasses of MHC-I proteins: classical and non-classical. Classical MHC-I proteins are
444 highly polymorphic, expressed by most nucleated cells, and present peptides derived
445 from intracellular proteins to $CD8^+$ cytotoxic T cell. Non-classical MHC-I proteins are
446 oligomorphic and the expression pattern of these proteins is limited to a few types of
447 tissues including the trophoblast (for a review see Rodgers and Cook, 2005). Even though
448 the function of non-classical MHC-I proteins has only been described in a few species, it
449 is generally accepted that the protection of the conceptus is a common function of these
450 proteins particularly among eutherian mammals (Ellis *et al.*, 1986; Comiskey *et al.*, 2003;

Hunt *et al.*, 2005).

The third possibility is that sheep are more sensitive to immunological challenges than goats. Roth *et al.* (1991) showed that the proliferation of sheep lymphocytes was suppressed more than goat lymphocytes when these cells were treated with trophoblast tissue-conditioned medium thus suggesting that sheep pregnancies are more dependent on conceptus derived signals for survival than goat pregnancies. It is reasonable to propose that sheep SCNT concepti are deficient in expressing immunosuppressive factors, which play a critical role in mediating maternal immuno-tolerance.

The retrospective nature of this study was not permissive for controlling for the source of fibroblasts for SCNT (fetal vs. adult). Although it has been postulated that donor cell type affects cloning efficiency, direct comparisons of nuclear donor cells of different origins show no evidence for this (for a review see Oback, 2008). Additionally, (Hirasawa *et al.*, 2013) demonstrated that extraembryonic gene expression was relatively consistent across pregnancies generated by different somatic cell donor types (cumulus, neonatal Sertoli and fibroblast cells) in cloned mice.

This is the first study to investigate the local immunological and inflammatory aspects at the maternal-fetal interface in term pregnancies generated by SCNT in sheep and goats. Further studies investigating the immunology of the maternal-fetal interface in early and mid-term SCNT pregnancies are now warranted. We propose that faulty nuclear reprogramming of SCNT embryos contribute to an altered expression of immuno-modulatory fetal proteins by the trophoblast cells, which then promotes a cytokine imbalance at the maternal-fetal interface causing placental insufficiency, pregnancy loss and various other complications. A dysfunctional maternal-fetal immune

474 relationship may contribute to metabolic conditions that affect fetal, newborn and even
475 adult health and survival (Mcmillen and Robinson, 2005). This study reaffirms the
476 importance of adequate maternal immuno-tolerance that will sustain pregnancy and result
477 in the birth of a normal, healthy neonate. These data could be used not only to improve
478 the outcomes of SCNT but also to understand the underlying mechanisms involved in
479 placental insufficiency and embryonic loss in livestock and humans.
480
481 Declaration of Interest
482 The authors declare that there is no conflict of interest that could be perceived as
483 prejudicing the impartiality of the research reported in this study.
484
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Table 1. Primers used for real time RT-PCR.

Gene	GenBank accession number	Primer sequence
GAPDH	U85042, AJ000039, AF022183, J04038	FP: GAGAAGGCTGGGGCTCACTT RP: GCTGACAATCTTGAGGGTGTTG
ACTB	AY141970	FP: GGCCGAGCGGAAATCG RP: GCCATCTCCTGCTCGAAGTC
IL1A	M37211	FP: GCCTTCAATAACTGTGGAACCAAT RP: GTATATTTCAGGCTTGGTGAAAGGA
IL2	M12791, M13204, X17201	FP: GCTGGATTTACAGTTGCTTTTGGAG RP: GATGTTTCAATTCTGTAGCGTTAACC
IL4	M77120, U14131, U14159, U14160	FP: GGCGTATCTACAGGAGCCACAC RP: CAAGAGGTCTTTCAGCGTACTTGT
IL5	Z67872	FP: TGGTGGCAGAGACCTTGACA RP: GAATCATCAAGTTCCCATCACCTA
IL6	X57317, X62501	FP: GGCTCCCATGATTGTGGTAGTT RP: GCCCAGTGGACAGGTTTCTG
CXCL8	AF232704, S74436	FP: GGAAAAGTGGGTGCAGAAGGT RP: GGTGGTTTTTTCTTTTTCATGGA
IL10	U00799	FP: GAGCAAGGCGGTGGAGAAGG RP: GATGAAGATGTCAAACTCACTCATGG
IL12B	U11815	FP: GCTGGGAGTACCCTGACACG RP: GGCTGAGGTTTGGTCCATGAAG
IL13	AJ132441	FP: CAGTGTCATCCAAAGGACCAAG RP: CGGACGTACTCACTGGAAACC
IL15	U42433	FP: GGGCTGTATCAGTGCAAGTCTTC RP: ATTGGGATGAGCATCACTTTCAG
IL17A	AF412040	FP: CATCATCCCACAGAGTCCAGG RP: CACTTGGCCTCCCAGATCAC
IL18	AF124789	FP: ACTGTTCAGATAATGCACCCCAG RP: GAAACAATTTTGTTCTCACAGGAGAG
IL23A	XM_588269	FP: CCTCCTTCTCCGTCTCAAGATC CGGAGGTCTGGGTGTCATCCT
IFNG	M29867, Z54144	FP: GATAACCAGGTCATTCAAAGGAGC RP: GATCATCCACCGGAATTTGAATC
TNF	Z48808, Z14137	FP: TCTACCAGGGAGGAGTCTTCCA RP: GTCCGGCAGGTTGATCTCA
TGFB1	M36271	FP: CTGAGCCAGAGGCGGACTAC RP: TGCCGTATTCCACCATTAGCA
CSF2	U22385	FP: CAGAAGTGGAAGCTTACCTCACAGA RP: CCTCCAGTGTGAAGATCCTGAGTT
IL2RA	NM_174358	FP: GCAGGGACCACAAATTTCCA RP: GTACTCAGTGGTAAATATGAACGTATCC
CD28	X93304	FP: GGAGGTCTGTGCTGTGAATGG RP: CGGTGCAGTTGAATTCCTTATTT

CTLA4	X93305	FP: GCAGCCAGGTGACCGAAGT RP: TCATCCAGGAAGGTTAGCTCATC
GATA3	XM581415, XM_864421, XM_872964, XM_873167, XM_873270, XM_873370	FP: CCGTGGTGTCTGTGTTCTCACT RP: TCAATAGGGAATGTGAGTCTGAATG
TBX21	XM_583748	FP: GGACACTGAAGCCCAGTTTTATAAC RP: CCAACCTAACGACATTCTTCCTGT
GNLY	AY245798	FP: GACAAGTTGGGAGATCAGCCC RP: ACCTACTGGCTTGCTTTTGCA
MHCI	EF569216 AJ874681.2	FP: GTGAGGTCACCCTGAGG RP: TGCTCCTCTCCAGAAGGCA
IFNA2	HQ585524	FP: GCACTGGATCAGCAGCTCACTG RP: CTCATGACTTCTGCTCTGACAACCT

790

791

Table 2. SCNT pregnancy rates and complications in sheep and goats.

	Number of transfers	Pregnancy rate, 45 days (%) [a]	Pregnancy rate, 60 days (%) [b]	Pregnancy rate, term (%) [b]	Pregnancy loss, 45 days to term (%) [c]	Large offspring syndrome (%) [d]
Sheep	82	32.9 (27/82)	19.5 (16/82)	19.5 (16/82)	40.7 (11/27)	31.3 (5/16)
Goat	37	32.4 (12/37)	32.4 (12/37)	32.4 (12/37)	0 (0/12)	0 (0/12)

[a] Pregnancy rates were similar ($P = 0.38$) at 45 days of gestation between sheep and goat SCNT generated embryos.

[b] SCNT pregnancy rates were greater ($P = 0.042$) in goat compared with sheep pregnancies at 60 days and term.

[c] SCNT pregnancy losses between 45 days and term were greater ($P < 0.001$) in sheep compared with goats.

[d] Incidence of large offspring syndrome was greater ($P < 0.001$) in sheep SCNT than in goat SCNT generated offspring.

Figure captions

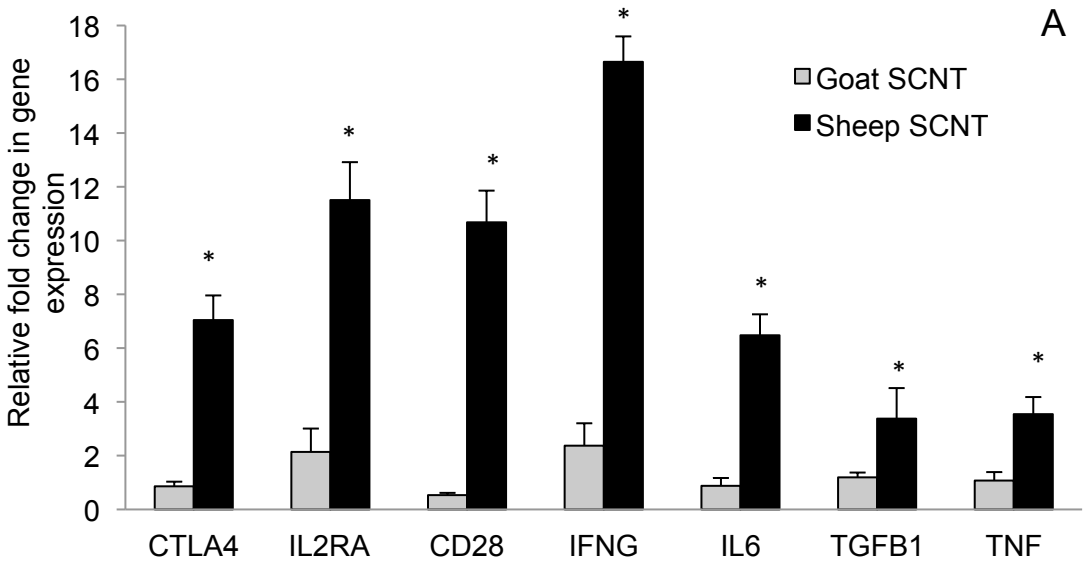
Figure 1. Fold change of gene expression of A. CTLA4, IL2RA, CD28, IFNG, IL6, TGFB1, TNF and; B. IL1A and CXCL8 in the intercotyledonary region of caprine and ovine placentas at term relative to placentas of pregnancies originated from natural breeding. Stars (*) indicate significant differences ($P \leq 0.05$) between SCNT pregnancies and the respective control group.

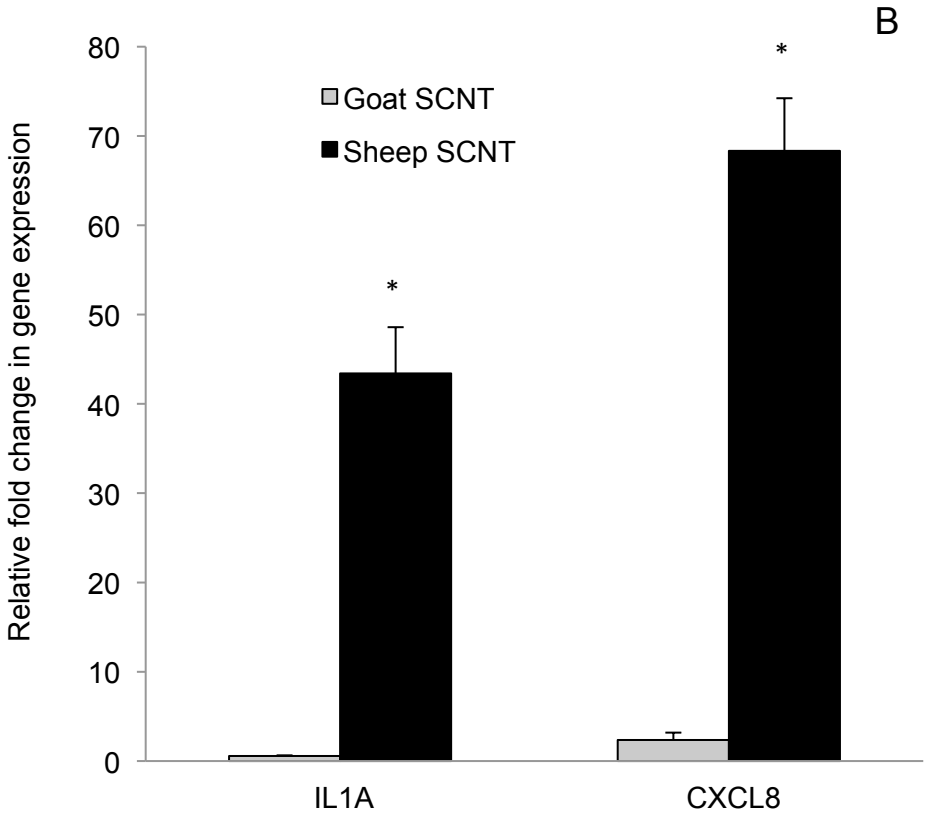
Figure 2. Fold change of gene expression in the cotyledonary region of term placentas from sheep and goat SCNT generated pregnancies relative to placentas of pregnancies originated from natural breeding. Stars (*) indicate significant differences ($P \leq 0.05$) between SCNT and the respective control group.

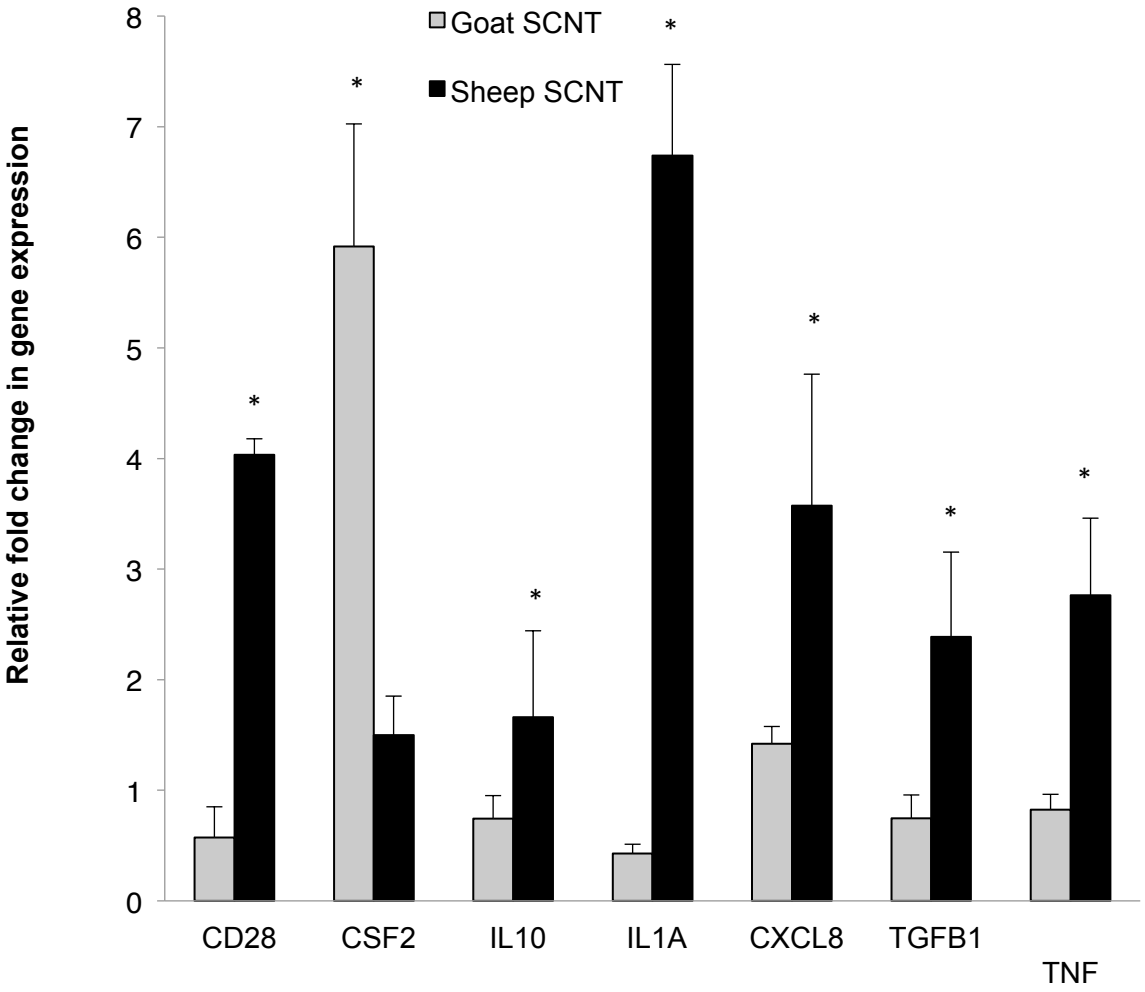
Figure 3 A. Fold change of gene expression relative to the expression of housekeeping genes (fold change of delta Ct) in the intercotyledonary region of term placentas from sheep and goat pregnancies established by natural breeding (control pregnancies). B. Fold change of gene expression relative to the expression of housekeeping genes (fold change of delta Ct) in the cotyledonary region of term placentas from sheep and goat control pregnancies. There was no statistical difference ($P > 0.05$) in gene expression between sheep and goat control pregnancies.

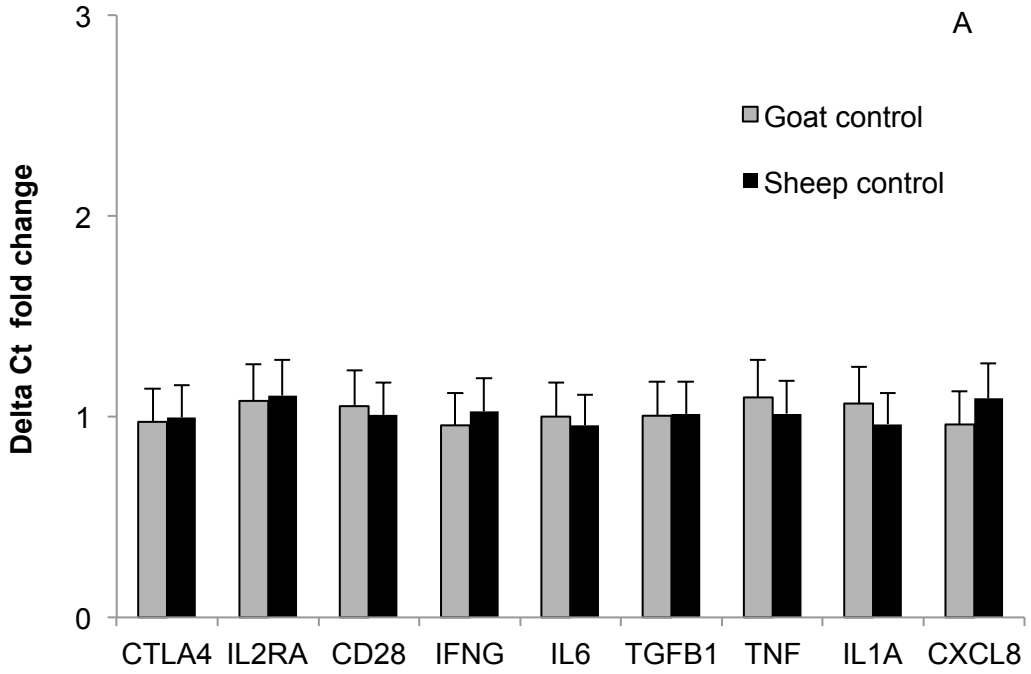
Figure 4.A. Fold change of MHC-I gene expression in the intercotyledonary region of term placentas from sheep and goat SCNT generated pregnancies relative to placentas of pregnancies originated from natural breeding. Stars (*) indicate significant differences (P

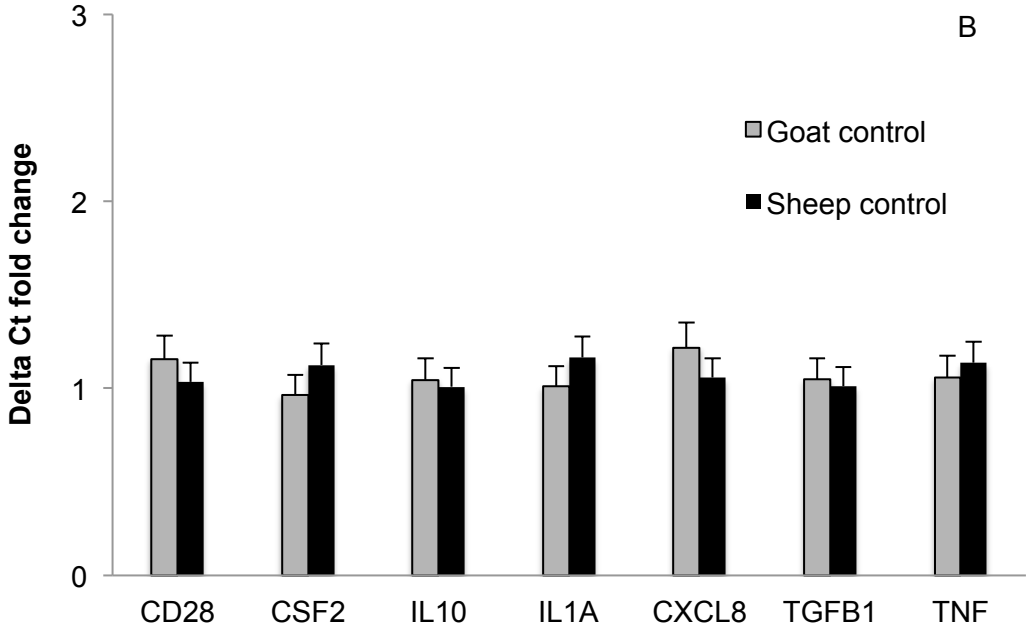
≤ 0.05) between SCNT and the respective control group. B. Immunohistochemical labeling of intercotyledonary trophoblast cells for MHC-I in the placenta of goat and sheep SCNT pregnancies, and goat and sheep pregnancies established by natural breeding (control groups). Scale bar = 50 μm.

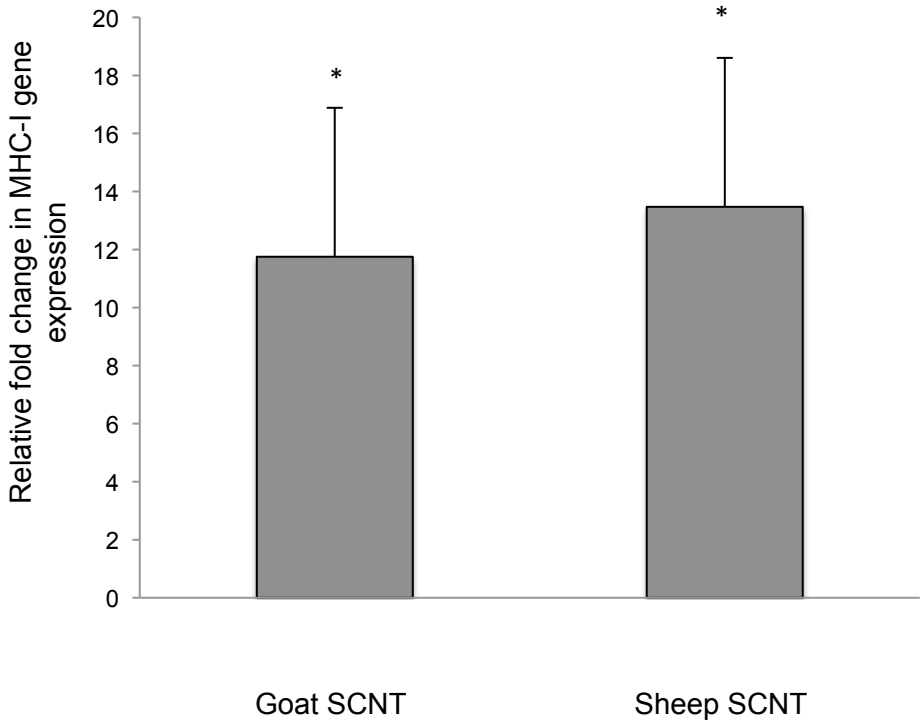

A
Relative fold change in gene expression
18
16
14
12
10
8
6
4
2
0
Goat SCNT
Sheep SCNT
CTLA4
IL2RA
CD28
IFNG
IL6
TGFB1
TNF

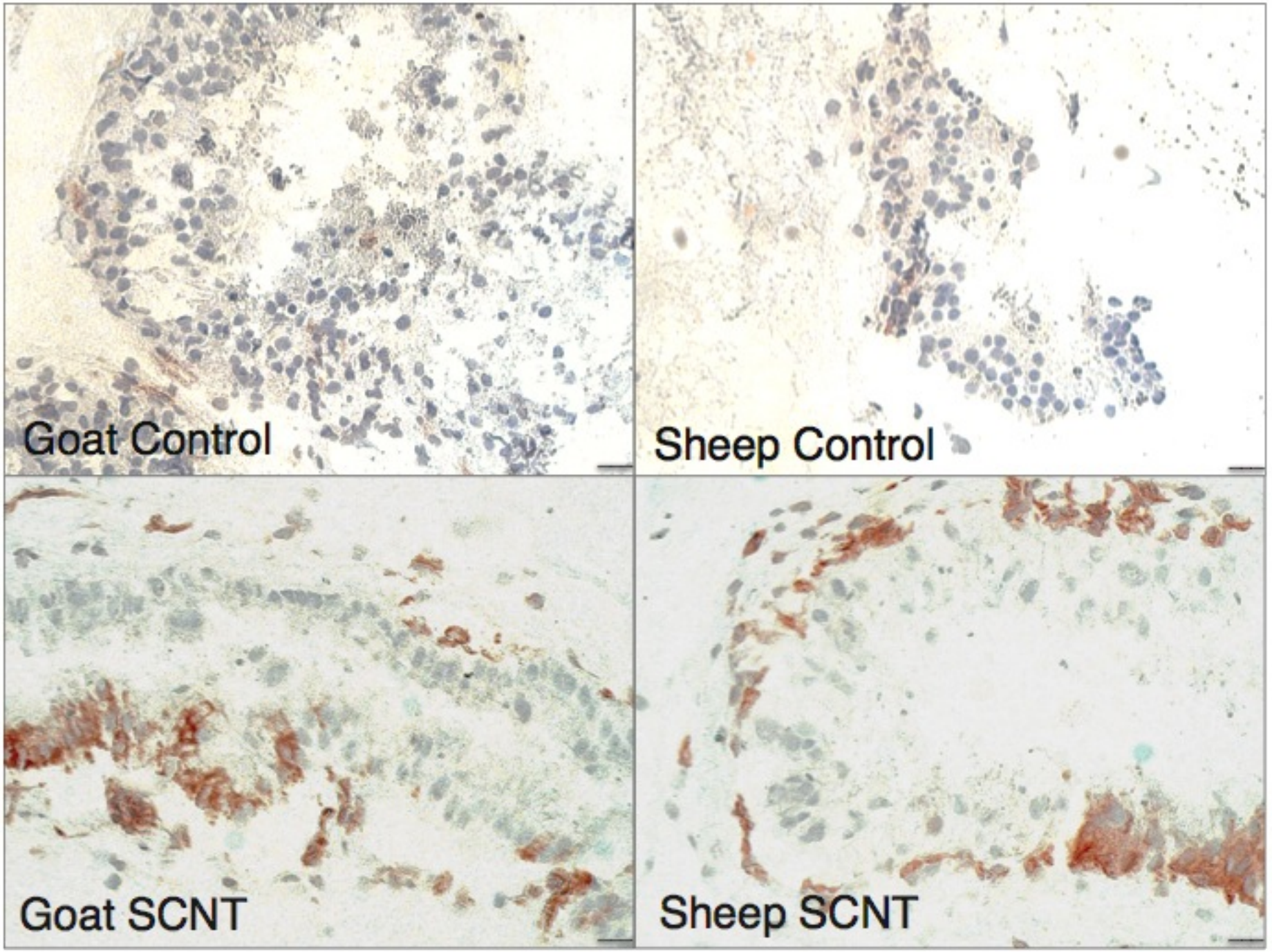

B
Relative fold change in gene expression
80
70
60
50
40
30
20
10
0
Goat SCNT
Sheep SCNT
IL1A
CXCL8


Relative fold change in gene expression
Goat SCNT
Sheep SCNT
0
1
2
3
4
5
6
7
8
*
CD28
CSF2
IL10
IL1A
CXCL8
TGFB1
TNF


A
Goat control
Sheep control
Delta Ct fold change
3
2
1
0
CTLA4
IL2RA
CD28
IFNG
IL6
TGFB1
TNF
IL1A
CXCL8


B
Goat control
Sheep control
Delta Ct fold change
3
2
1
0
CD28
CSF2
IL10
IL1A
CXCL8
TGFB1
TNF


Relative fold change in MHC-I gene expression
20
18
16
14
12
10
8
6
4
2
0
*
*
Goat SCNT
Sheep SCNT


Goat Control
Sheep Control
Goat SCNT
Sheep SCNT