

Imprinted gene expression in *in vivo*- and *in vitro*-produced bovine embryos and chorio-allantoic membranes

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ABSTRACT. Cloning by nuclear transfer is often associated with poor results due to abnormal nuclear reprogramming of somatic donor cells and altered gene expression patterns. We investigated the expression patterns of imprinted genes *IGF2* and *IGF2R* in 33- to 36-day bovine embryos and chorio-allantoic membranes derived from *in vivo*- and *in vitro*-produced embryos by somatic cell nuclear transfer (SCNT), parthenogenetic activation, and *in vitro* fertilization (IVF). There was a lower *IGF2* expression rate in the SCNT (0.19) and parthenogenetic (0.02) groups when compared to *in vivo* and IVF embryos (2.01; $P < 0.05$). In the chorio-allantoic membranes, *IGF2* showed a baseline expression pattern ($P < 0.05$) in parthenotes (0.001) when compared to *in vivo*, IVF (3.13), and SCNT (0.98) groups. *IGF2R* was less expressed ($P < 0.05$) in SCNT chorio-allantoic membranes (0.25) when compared to the *in vivo* group. The low expression of *IGF2* in parthenogenetic embryos and

chorio-allantoic membranes confirms its imprinted status in cattle. Alterations in the relative frequency of *IGF2* and *IGF2R* transcripts were observed in SCNT-derived bovine embryos and chorio-allantoic membranes, respectively, supporting the hypothesis that abnormalities in the expression of imprinted genes are causes of the low efficiency of SCNT procedures in this species.

Key words: Cattle; Epigenetics; Genomic imprinting; *IGF2*; *IGF2R*; Nuclear transfer

INTRODUCTION

The limited success of somatic cell nuclear transfer (SCNT) is often related to incorrect nuclear reprogramming of donor cells. There were two epigenetic mechanisms underlying this process: DNA methylation and histone remodeling. Both are involved in the control of several cellular processes, such as the control of gene expression, X inactivation, and genomic imprinting (Li et al., 1993; Holmes and Soloway, 2006).

Genomic imprinting can be defined as the unequal contribution of parental genomes during development. Imprinted genes play important roles in the development of the fetus and placenta, and some alterations associated with reproductive technologies are believed to be caused by failures in imprinted gene regulation. However, despite the large use of these biotechniques in cattle, there are few studies focusing on these genes in cattle.

The present study was designed to compare the relative frequency of messenger RNA (mRNA) of the imprinted genes *IGF2* (paternally expressed) and *IGF2R* (maternally expressed) in bovine embryos and chorio-allantoic membranes obtained *in vivo* or by *in vitro* fertilization (IVF), SCNT, and parthenogenesis.

MATERIAL AND METHODS

Chemicals

Chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated.

In vivo embryo production

Embryos on days 33 to 36 of development and chorio-allantoic membranes from *in vivo*-produced embryos were obtained after superovulation and artificial insemination of cycling *Bos taurus* x *B. indicus* crossbreed cows. Follicular waves were synchronized by dominant follicle aspiration and insertion of a progesterone (P4) releasing device (Crestar, Intervet Internation GmbH, Unterschleissheim, Germany). Two hundred milligrams FSH (Folltropin™, Bioniche Animal Health, Belleville, Canada) was administered *im* in eight decreasing doses, twice a day, for four consecutive days (40 mg x 2; 30 mg x 2; 20 mg x 2, and 10 mg x 2), beginning 3 to 4 days after follicular aspiration. To induce luteolysis, along with the fifth FSH injection, a dose of 150 µg prostaglandin F2α analogue (D-cloprostenol; Preloban,

Intervet International GmbH) was administered *im*. Removal of the P4 releasing device was performed together with the sixth FSH injection. Finally, 0.1 mg gonadotrophin-releasing hormone (GnRH; Fertagyl, Intervet International GmbH) was administered *im* along with the eighth FSH injection in order to induce LH surge. Cows were inseminated 12 and 24 h post-GnRH application with Nelore (*Bos indicus*) sire semen.

***In vitro* embryo production**

Abattoir-derived ovaries from crossbreed cows were transported to the laboratory in saline solution at 28–32°C. Follicles (3 to 8 mm) were aspirated using an 18-G needle attached to a 20-mL syringe. Oocytes with at least four layers of cumulus cells (COCs) and homogeneous cytoplasm were selected for *in vitro* maturation. Groups of 20 to 25 oocytes were matured under mineral oil (Dow Corning Co., Midland, MI, USA) in droplets (100 µL) of tissue culture medium (TCM-199, Gibco BRL, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated (55°C for 30 min) fetal bovine serum (FBS), 1.0 µg/mL FSH (Folltropin™), 50 µg/mL hCG (Profasi™, Serono, São Paulo, Brazil), 1.0 µg/mL estradiol, 0.20 mM sodium pyruvate, and 83.4 µg/mL amikacin (Instituto Biochimico, Rio de Janeiro, Brazil). Oocytes were maintained in an atmosphere of 5% CO₂ in air at 38.5°C for 24 h.

***In vitro* fertilization**

To produce embryos by IVF, at 24 h post-maturation, expanded COCs were transferred to 100 µL drops of TALP-IVF medium supplemented with 6 mg/mL bovine serum albumin (BSA), 30 µg/mL heparin, 18 µM penicillamine, 10 µM hypotaurine, 1.8 µM epinephrine, and 0.2 mM sodium pyruvate. Semen from a Nelore bull was used, and motile spermatozoa were separated by density gradient in 45 and 90% (v/v) Percoll. Final sperm concentration used was 1×10^5 live spermatozoa per drop. Oocytes and sperm cells were co-incubated for 22 h at 38.5°C under 5% CO₂ in air and maximum humidity. At the end of co-culture, presumptive zygotes were washed in TALP-IVF medium and transferred to embryo culture medium.

Somatic cell nuclear transfer

The somatic cell line used as nucleus donor was derived from a skin biopsy obtained from a 19-year-old Nelore cow (*Bos indicus*). The biopsy was cut into small pieces (3 mm) and the explants were cultured in 25-mm² flasks containing Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 50% (v/v) FBS and 83.4 µg/mL amikacin at 38.5°C under an atmosphere of 5% CO₂ in air and maximum humidity. This medium was used during the first 3 days of culture for primary cell line establishment. Cell culture was then maintained using DMEM plus 10% (v/v) FBS and 83.4 µg/mL amikacin. When primary cell line confluence was achieved, cells were treated with 0.05% (w/v) trypsin supplemented with 1% (v/v) poultry serum and replated. All passages were cultured until confluence. Cells from early passages were frozen in DMEM supplemented with 10% (v/v) dimethyl sulfoxide (Merck & Co. Inc., Whitehouse Station, NJ, USA) and stored in liquid nitrogen. Thawed cells, cultured for 3 to 4 passages, were used in nuclear transfer procedures as nucleus donors.

At 18 h post-maturation, expanded COCs were placed in 0.2% (w/v) hyaluronidase

for 5 min and gentle pipetting was performed to remove the cumulus cells. Oocytes showing the first polar body were selected and incubated in synthetic oviductal fluid (SOF) with 10 µg/mL Hoechst 33342 stain and 7.5 µg/mL cytochalasin B at 38.5°C under 5% CO₂ in air and maximum humidity. Enucleation was performed in HEPES-buffered SOF (HSOF; Wells et al., 1999) supplemented with 10% (v/v) FBS and 7.5 µg/mL cytochalasin B, with a 25-µm (external diameter) glass pipette, by removing the first polar body and the surrounding cytoplasm. Enucleation was confirmed by visualizing the aspirated chromatin inside the pipette under ultraviolet light. After enucleation, cytoplasts were washed twice in HSOF + 10% (v/v) FBS and held in this medium until reconstruction. Individual fibroblast cells were transferred into the perivitelline space of each recipient oocyte in HSOF containing 10% (v/v) FBS and 7.5 µg/mL cytochalasin B. The oocyte-cell complexes were fused in 0.28 M mannitol solution containing 0.05 mM Ca²⁺, 0.1 mM Mg²⁺, and 0.3 mg/mL BSA. Cell fusion was induced with two direct pulses of 2.0 kV/cm for 20 µs each, using a BTX Electrocell Manipulator 2001 (BTX, San Diego, CA, USA). Fusion rates were determined 30 to 60 min after electrofusion, and successfully reconstructed oocytes were incubated in HSOF + 10% (v/v) FBS at 38.5°C under a humidified atmosphere of 5% CO₂ in air until activation.

Reconstructed oocytes were chemically activated 30 h post-maturation, using 5 µM ionomycin for 5 min in HEPES-buffered TCM-199 supplemented with 10% (v/v) FBS, followed by incubation in SOF supplemented with 20 mM strontium chloride (SrCl₂·6H₂O; Mallinckrodt, Hazelwood, MO, USA) and 10 µg/mL cytochalasin B for 6 h at 38.5°C under a humidified atmosphere of 5% CO₂ in air (Yamazaki et al., 2005).

Parthenogenetic embryos

To obtain parthenogenetic blastocysts, oocytes matured for 24 h were denuded, selected for the first polar body presence, and kept in maturation medium until activation. At 30 h post-maturation, oocytes were activated with ionomycin followed by incubation in strontium supplemented with cytochalasin B, as described previously for activation of reconstructed oocytes.

Embryo *in vitro* culture and embryo transfer

Embryo culture was performed in 100-µL microdrops of modified SOF (Vajta et al., 1999) containing 5 mg/mL fatty acid-free BSA and 2.5% (v/v) FBS, overlaid with mineral oil. Groups of 10 to 20 embryos were cultured together in each microdrop at 38.5°C under a humidified atmosphere of 5% CO₂ in air for 7 days, until the blastocyst stage.

Crossbreed *B. indicus* x *B. taurus* cows were synchronized with 150 µg D-cloprostenol, and females showing estrus received 7 blastocysts produced by IVF, SCNT or parthenogenetic activation. On day 30 of gestation, the presence of the conceptus was determined by *per rectum* ultrasound examination. Surrogate cows with positive diagnosis were slaughtered between days 33 and 36 of pregnancy.

Recovery of conceptus and embryonic membranes

Immediately after slaughter of pregnant cows, the reproductive tract was removed, placed on ice, and transported to the laboratory. The uterine horns were dissected

by cutting along the major curvature, exposing the conceptus and embryonic membranes. The entire embryos and fragments of chorio-allantoic membranes were collected, frozen in liquid nitrogen, and stored at -80°C . A total of eleven embryos were recovered, two in the SCNT group and three in each of the *in vivo*, IVF, and parthenogenetic groups.

Analysis of gene expression

RNA extraction of *in vivo*-, IVF-, SCNT-, and parthenogenetic-derived 33- to 36-day embryos and chorio-allantoic membranes was performed using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following manufacturer instructions.

For reverse transcription, 1 μg total RNA was incubated for 5 min at 70°C with 6.75 μM oligo-dT (pd(T)₁₂₋₁₈; Amersham Biosciences, Piscataway, NJ, USA), and then immediately cooled at 4°C before the addition of 50 U reverse transcriptase enzyme (ImProm-II™ Reverse Transcriptase, Promega, Madison, WI, USA) and reagent mix: buffer - 250 mM Tris, 375 mM KCl, 50 mM DTT, 3 mM MgCl₂, 0.5 mM of each dNTP (Invitrogen), and 2 U/ μL of RNase inhibitor (Amersham Biosciences) in a final volume of 20 μL . Samples were heated to 42°C for 1 h, incubated at 70°C for 15 min, and frozen at -20°C until use.

Amplifications were performed in a real time polymerase chain reaction (PCR) thermocycler (Applied Biosystems 7500 Real Time PCR System, Applied Biosystems, Foster City, CA, USA) using Power SYBR Green® PCR Master Mix (Applied Biosystems), 0.06 μM , 0.20 μM , and 0.50 μM of each pair of primer for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *IGF2*, and *IGF2R*, respectively, in a 20- μL reaction. The reaction was initiated with incubation at 50°C for 2 min, followed by denaturation at 95°C for 10 min, and 45 cycles at 95°C for 30 s and 60°C for 1 min. Sequences of primers for imprinted and housekeeping (*GAPDH*) genes are shown in Table 1.

Table 1. Sequence of primers used for amplification of imprinted (*IGF2* and *IGF2R*) and housekeeping (*GAPDH*) genes, and GenBank code.

Gene	Sequence (5'→3')	GenBank
<i>IGF2</i>	CTTCAGCCGACCATCCAGCCGCATAAAC TCAGCGGACGGTGACTCTTGGCCTCTCT	X53553
<i>IGF2R</i>	CGCCTACAGCGAGAAGGGGTTAGTC AGAAAAGCGTGCACGTGCGCTTGTC	J03527
<i>GAPDH</i>	GCGTGAACCACGAGAAGTATAA CCCTCCACGATGCCAAAGT	AB098979

The PCR efficiency was estimated for *GAPDH* and imprinted genes in each sample using a method and software published previously (Ramakers et al., 2003) applying default parameters (number of points between 4 and 6 and best correlation coefficient). For each pair of primers, the fluorescence threshold line was fixed at the average of the lower and higher fluorescence values used by the software to estimate PCR efficiency.

Fold differences in amount of *GAPDH* or target genes mRNA were calculated by the equation: fold change = $[1 + (\text{average PCR efficiency})]^{\text{average Ct test} - \text{average Ct reference}}$ (Livak and Schmittgen, 2001).

To measure the differences, we used the pair-wise fixed reallocation randomization test in the Relative Expression Software Tool (REST; Pfaffl et al., 2002). The hypothesis of no difference between groups was the null one (H_0) and the differences were considered to be statistically significant when the probability of the alternative hypothesis [$P_{(H1)}$] was less than 0.05.

RESULTS

The comparisons of imprinted gene expression in 33- to 36-day embryos produced *in vivo* or *in vitro* demonstrated a lower expression ($P < 0.05$) of *IGF2* in SCNT (0.19) and parthenogenetic-derived samples (0.02) when compared to *in vivo* and IVF (2.01; Figure 1). However, no differences were observed ($P > 0.05$) among groups in *IGF2R* gene expression (Figure 1).

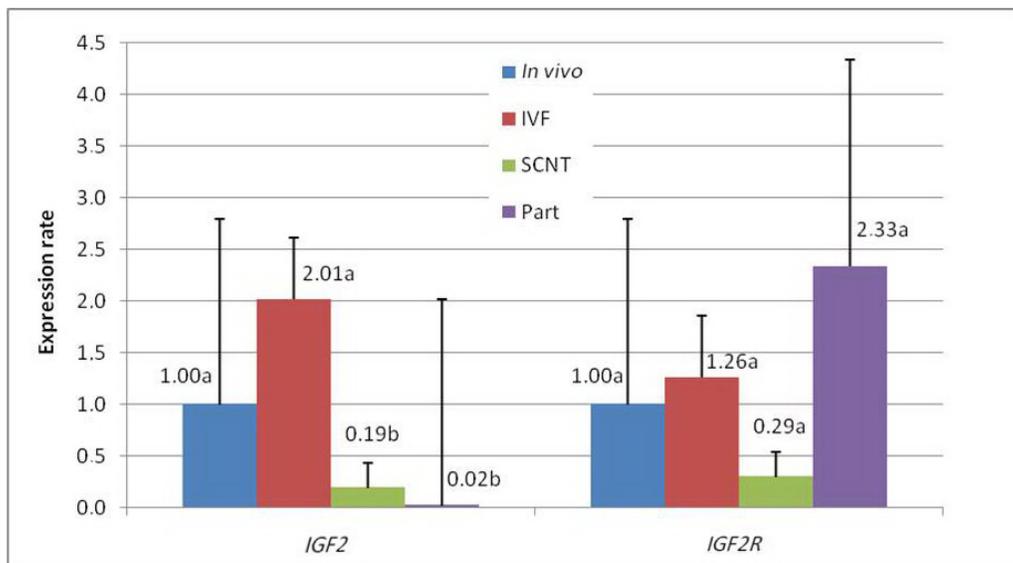


Figure 1. Rate of expression of *IGF2* and *IGF2R* in embryos derived from *in vivo*- or *in vitro*-produced blastocysts [*in vitro* fertilization (IVF), somatic cell nuclear transfer (SCNT), and parthenogenetic (Part) groups]. Values followed by different letters differ significantly ($P < 0.05$). Reference group = *in vivo*.

The analysis of chorio-allantoic membranes of the bovine conceptus revealed a baseline expression pattern ($P < 0.05$) in the *IGF2* gene in parthenogenetic-derived samples (0.001) when compared to *in vivo*, IVF (3.13), and SCNT (0.98). A lower expression was observed ($P < 0.05$) in *IGF2R* gene in SCNT-derived samples (0.25) when compared to the IVF group (1.21; Figure 2).

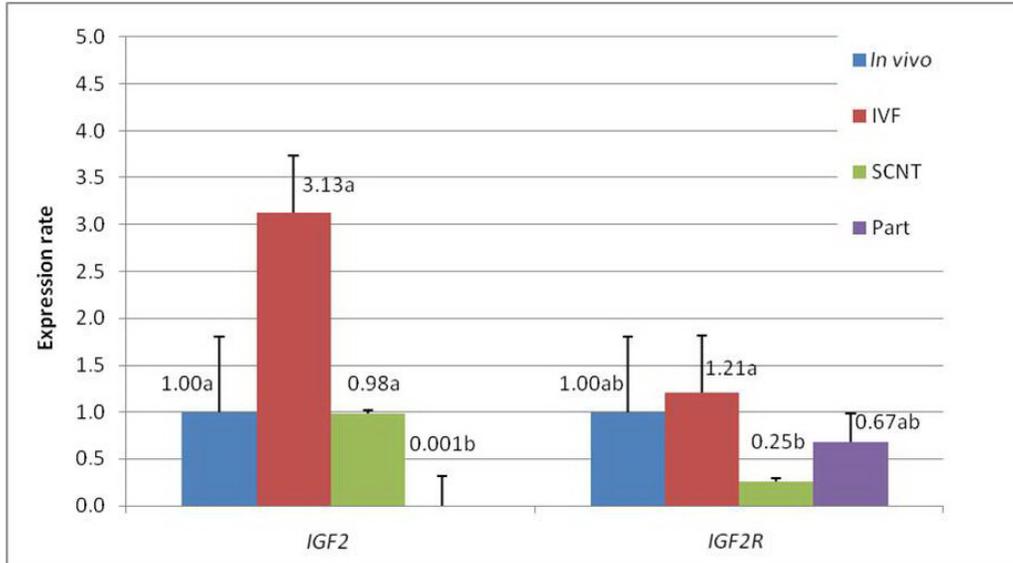


Figure 2. Rate of expression of *IGF2* and *IGF2R* in chorio-allantoic membranes of conceptus derived from *in vivo*- or *in vitro*-produced blastocysts [*in vitro* fertilization (IVF), somatic cell nuclear transfer (SCNT), and parthenogenetic (Part) groups]. Values followed by different letters differ significantly ($P < 0.05$). Reference group = *in vivo*.

DISCUSSION

About 112 genes or non-coding RNAs are known to be imprinted in humans and mice, and some of these are conserved in ruminants (Reik and Walter, 2001; Young et al., 2003; Morison et al., 2005). Dozens of them are involved in fetal growth and development, and are particularly important to normal placenta development and nutrient transport from placenta to fetus, especially amino acids and glucose (Tilghman, 1999; Constância et al., 2002). In general, maternally derived imprinted genes reduce growth while paternally derived imprinted genes promote growth, and this occurs both in fetal and placental tissues.

The maintenance of differential patterns of methylation, at the differentially methylated regions (DMR) of alleles, is determinant to monoallelic expression of imprinted genes. Therefore, the loss of DNA methylation at DMR leads to *IGF2* and *IGF2R* being normally expressed by paternal and maternal alleles, respectively, to become silenced, while *H19*, normally expressed from the maternal allele, shows biallelic expression (Li et al., 1993).

The *IGF2* gene codes for a growth factor involved in fetal and placental development. In humans, the *IGF2* gene is related to the Silver-Russell syndrome (SRS), characterized by the lack of methylation at the *IGF2-H19* imprinted control region (ICR), lower *IGF2* expression, and biallelic expression of *H19*, with intrauterine and postnatal growth retardation (Blik et al., 2006). Oppositely, the overexpression of *IGF2* in humans causes the Beckwith-Wiedemann syndrome (BWS), characterized by biallelic methylation of *IGF2-H19* ICR, lack of *H19* expression, and biallelic expression of *IGF2*. The individual affected by BWS shows pre- and postnatal overgrowth, organomegaly and neonatal hypoglycemia (Delaval et al., 2006). Similarly, the

overexpression of *Igf2* in mice promotes fetal overgrowth (Eggenchwiler et al., 1997), while underexpression of *Igf2* was associated with a deficit in growth (DeChiara et al., 1990).

In cattle, the use of IVF and cloning raised the incidence of offspring with symptoms that resemble BWS or SRS, such as the large offspring syndrome (LOS) or the retardation of development, commonly seen in animals produced by reproductive biotechnologies (De-Baun et al., 2003; Mann et al., 2003; Young et al., 2003). Overexpression of *IGF2* has been described in cardiac and hepatic tissues of cloned calves (Li et al., 2007) with neonatal death, as well as in renal tissues of cloned mice (Humpherys et al., 2002). Abnormal expression of *Igf2* was also described in the fetus and placenta of cloned mice (Ogawa et al., 2003) or in placentas of cloned calves after birth (Yamazaki W, Perecin F, Méo SC, Ferreira CR, et al., unpublished data) with lower expression rates in both cases.

In our results, similar expression of *IGF2* between IVF and *in vivo* contrasted with decreased expression in SCNT group in embryonic tissues. In chorio-allantoic membranes, similar expression of *IGF2* between *in vivo*, SCNT, and IVF groups was observed. The almost complete absence of *IGF2* transcripts in embryonic tissues as well as in the chorio-allantois derived from parthenogenesis supported its maternal imprinted status in ruminants. The placental phenotype and its capacity to transfer substances from the mother to the fetus depend on the interplay between placental and fetal *IGF2*, with direct influence on fetus growth (Coan et al., 2008). The ratios between placental and embryonic *IGF2* expression rates were 1.0, 1.5 and 5.1 for *in vivo*, IVF and SCNT, respectively, indicating a pronounced imbalance of *IGF2* in cloned-derived tissues.

The *IGF2R* gene, paternally imprinted, also plays an important role as negative effector in fetal growth since it promotes *IGF2* arrest into lysosomes followed by degradation (Lau et al., 1994; Ludwig et al., 1996). In sheep, parthenogenetic embryos express low levels of *IGF2* and high levels of *IGF2R* and *H19*, and have retarded growth when compared to control embryos (Feil et al., 1998; Hagemann et al., 1998; Young et al., 2003). Conversely, the absence of *IGF2R* expression in ovine embryos submitted to *in vitro* culture is associated with LOS (Young et al., 2001), demonstrating that the culture conditions affect the imprinting status of *IGF2R*.

We observed lower *IGF2R* expression in chorio-allantoic membranes derived from embryos produced by SCNT. Long and Cai (2007) examining cloned bovine fetuses that suffered perinatal death found aberrant patterns of DNA methylation at *IGF2R* ICR, as did Young et al. (2003) who observed loss of methylation in *IGF2R* DMR of cloned sheep. Similar observations in cloned murine fetuses were described by Ogawa et al. (2003). Reduced expression of *IGF2R* was described in cardiac, hepatic, and renal tissue of cloned calves with neonatal death (Li et al., 2007), contrasting with the results described by Yang et al. (2005), in which no alteration in *IGF2R* expression was found in dead neonatal cloned calves. However, despite that hypomethylation in *IGF2R* DMR has been frequently reported, alterations in *IGF2R* expression in the placenta of cloned animals that developed to term have never been found.

Given that *IGF2R* acts as a clearance receptor for *IGF2*, the balance between the two genes is important to regulate the effects of *IGF2*. Young et al. (2001) showed reduced expression of *IGF2R* in cloned fetuses with overgrowth, assuring that the lack of *IGF2* clearance may give rise to LOS. In our results, although some variation was seen in the ratio *IGF2/IGF2R* in both IVF and SCNT embryos and in IVF chorio-allantois, a striking imbalance was observed in SCNT chorio-allantois due to lower expression of *IGF2R*. Functional and morphological deficiencies in the placenta are commonly observed in gestations of cloned animals (Farin et

al., 2006; Miglino et al., 2007). Frequently, these alterations include placental overgrowth that occurs before fetal overgrowth (Constant et al., 2006), suggesting that some of the problems observed in cloned animals have their origin in the placenta and not in the conceptus.

Another gene that can affect *IGF2* post-transcriptionally is the *H19* gene (Runge et al., 2000). Located at the flanking position of *H19-IGF2* ICR, it gives rise to a non-coding RNA that acts through an unclear mechanism regulating the amount of the IGF-II protein. We also assessed the expression rates of *H19* in the same tissues as for *IGF2* and *IGF2R*, but no significant differences among groups were found (data not shown).

After fertilization, the distinct epigenetic marks between alleles are already established and are maintained throughout development. Therefore, it is unclear when the alterations observed in imprinted genes occur. Although a possible origin in the gametes cannot be discarded, several observations indicate that these failures frequently occur after fertilization, i.e., the presence of mosaicism in DNA methylation patterns is observed in monozygotic twins in which only one has imprinting abnormalities (Bailey et al., 1995). The epigenetic control of imprinted genes through DNA methylation provides a linking mechanism between environmental stimulus and the epigenotypes. Thus, together with the fact that animals produced by nuclear transfer are more prone to show signs associated with imprinted gene disruptions, the role of *in vitro* culture conditions or gamete manipulations in the ethiogenesis of these problems must be considered.

The significant alterations in the level of transcripts from imprinted genes in SCNT-derived embryos and placentas indicate that abnormal expression of imprinted genes is one of the causes for the low efficiency of cattle cloning. The alterations observed in embryos and placentas produced by SCNT are suggestive of modifications of DNA methylation at the ICRs of *IGF2* and *IGF2R*.

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