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Changes in the expression pattern of developmentally important genes and possible relation to the low *in vitro* and *in vivo* developmental potential of transgenic nuclear transfer embryos

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Abstract

The efficiency of nuclear transfer was evaluated *in vitro* and *in vivo* using non-transgenic and transgenic cloned bovine embryos. In addition, in order to better understand the low pre- and post-implantation development observed in transgenic nuclear transfer embryos, a gene expression data analysis was conducted in this group. We observed no difference ($P > 0.05$) in the cleavage rate of nuclear transfer embryos generated with transgenic and non-transgenic fetal fibroblasts. However, the blastocyst rate was affected ($P < 0.01$) in transgenic (13%) compared to non-transgenic nuclear transfer embryos (24%). Despite this difference, the quality of embryos as assessed by the total number of cells and morphological appearance was not different ($P > 0.05$). Pregnancy rate was also affected ($P < 0.05$) in transgenic (7%) compared to non-transgenic nuclear transfer embryos (43%), though all pregnancies failed to maintain to term. Gene expression data analysis revealed a decrease ($P < 0.05$) of *INF*-τ and *HDAC1* in transgenic nuclear transfer embryos relative to embryos generated by *in vitro* fertilization. Considering the importance of these two genes in maternal recognition of pregnancy and nuclear reprogramming, the alterations observed in these transgenic embryos might help to explain the low pre- and post-implantation development observed in this group, which highlights the need to assess different donor cells before embryo transfer, particularly when the aim is to generate transgenic offspring.

Keywords: cattle, cloning, embryo transfer, transgenic.

Introduction

Since the birth of the first cloned mammal by somatic cell nuclear transfer (SCNT; Wilmut *et al.*, 1997), the technology has been successfully replicated in a number of agricultural and domestic species. Furthermore, nuclear transfer has also been used to generate transgenic offspring (Schnieke *et al.*, 1997; Cibelli *et al.*, 1998; Brophy *et al.*, 2003; Echelard *et al.*, 2009; Nowak-Imialek *et al.*, 2011). In this case, prior to nuclear transfer, the cell used as the nuclear donor is genetically manipulated to target the desired modification in the animal.

In vitro development of SCNT has been improved from the first successful attempt in 1996 to the point that a similar rate of blastocysts to that of their IVF counterparts can be obtained (Wells *et al.*, 1999, 2003). Although a few studies in cattle report that a similar rate of pregnancy was observed compared to NT, IVF or *in vivo* derived embryos (Hill *et al.*, 2000; Heyman *et al.*, 2002), the efficiency of generating live offspring as a proportion of embryos transferred has remained low, as is the case in most species. In fact, the success rate in cattle, one of the species where more laboratories have succeeded in generating live offspring (Oback and Wells, 2003), ranges around 11% with adult cells (Kubota *et al*., 2000; Gibbons *et al*., 2002; Panarace *et al*., 2007), although with blastomeres as nuclear donors, efficiency rates up to 25% have been obtained (Peura and Trounson, 1998). For a small set of experiments, a much higher efficiency rate has also been observed (80%; Kato *et al*., 1998), but this is not representative of the majority of the experiments. A common characteristic in all transgenic and non-transgenic cloned animals has been the high incidence of abortion and mortality during pregnancy (Kato *et al.*, 1998; Hill *et al.*, 2000; Panarace *et al.*, 2007). High pregnancy losses occur during the first trimester of gestation (Hill *et al.*, 2000; Zakhartchenko *et al.*, 2001; Heyman *et al.*, 2002), although late gestation losses are also observed compared to IVF or *in vivo* derived pregnancies (Heyman *et al.*, 2002).

A number of factors may contribute to the low efficiency of generating live offspring, including the synchrony of the cell cycle stage of both the donor cell (Gibbons *et al.*, 2002) and recipient oocyte (Campbell *et al.*, 1994), donor cell origin (Kato *et al.*, 2000; Poehland *et al.*, 2007), donor cell age (Hill *et al.*, 2000; Lagutina *et al.*, 2005), donor cell passage number (Arat *et al.*, 2001), *in vitro* culture conditions of reconstructed embryos (Zakhartchenko *et al.*, 1999) and inadequate nuclear

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reprogramming (Stice and Robl, 1988). Of particular interest are the modifications that may occur during cell culture, which might result in epigenetic changes to the donor nucleus, making these cells more or less capable of being reprogrammed. In fact, wide-spread alterations in the gene expression profile of embryos and cloned animals have been observed, which suggests that transcriptional regulation mechanisms are impaired at different levels during the reprogramming process (Dean *et al.*, 2001; Beyhan *et al.*, 2007; Suzuki *et al.*, 2008). This is particularly important when using nuclear transfer technology for generating transgenic animals, since an extended period of culture is required for the selection of transgenic cells in the presence of a marker gene, a process that has been observed to have important effects on the nuclear transfer efficiency of these cells. In fact, contradictory reports have been published in different species regarding the developmental competence of nuclear transfer embryos generated with transgenic and non-transgenic cells of different origin (Ogura *et al.*, 2000; Arat *et al.*, 2001, 2002; Zakhartchenko *et al.*, 2001; Iguma *et al.*, 2005; Wang *et al.*, 2007; Zhang *et al.*, 2010). Consequently, in the present study we examined the *in vitro* and *in vivo* developmental potential of nuclear transfer embryos generated with transgenic and non-transgenic bovine fetal fibroblasts derived from the same source. In addition, we investigated the relative expression pattern of 20 developmentally important genes in transgenic nuclear transfer embryos using reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) in an attempt to assess if the low developmental and implantation rates observed with these embryos could be attributed to an aberrant gene expression pattern at the blastocyst stage.

Material and Methods

DNA construction

A gene expression vector containing the *Staphylococcus aureus* A protein gene (pMPA2) was constructed by inserting *SPA* genes (accession number: M18264) between DNA fragments containing a bovine beta-casein promoter and terminator regions into pCR-XL-TOPO® (Invitrogen, Carlsbad, CA, USA) vector. The pMPA2 plasmid was digested with *Sal*I and *Not*I and the DNA fragment containing the gene construction was gel-purified before transfection of donor cells.

Derivation, transfection of donor cells and chromosome analysis

A 4-year-old Holstein cow was inseminated with frozen/thawed semen from a Holstein bull of proven fertility. After 50 days, the female was sent to the slaughterhouse (Frigorifico Temuco) and the fetus recovered in the processing line and taken immediately to the laboratory in Dulbeccos´s PBS (GIBCO Invitrogen, Carlsbad, CA, USA) at 4ºC. Bovine fetal fibroblasts were isolated from the fetus and cell explants cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (HyClone Laboratories, Inc., Logan, UT, USA) at 38.5°C in a humidified atmosphere of 5% CO_2. When the cells reached confluency, a portion was frozen for future NT experiments (non-transgenic cells) and the rest were kept growing for transfection experiments. One day before transfection, 2 x 10^5 cells were plated onto a 35 mm culture dish and cultured for 16 h to achieve 60-80% confluency. Transfection was carried out by combining 2 µg MPA with Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were selected with 800 µg/ml Geneticin (G418, HyClone Laboratories, Inc., Logan, UT, USA) for 7-10 days and 7 single colonies isolated by ring cloning. Chromosome analysis was carried out on each clone, including the non-transgenic cells, for which cells were grown individually in DMEM media containing 10% FCS until confluency. Then, the clones were incubated with 0.5 µg/ml KaryoMax (GIBCO Invitrogen, Carlsbad, CA, USA) for 5-6 h at 39ºC and treated with a hypotonic solution of 0.9% Sodium citrate for 25 min at 39ºC. After that, the cells were fixed in methanol:acetic acid (3:1) and spread on clean microscopic slides. Chromosomes were stained with 5% Giemsa for 10 min and examined at 1000X magnification under oil to determine the chromosome number. This analysis allowed the selection of a transgenic clone with a normal chromosome number (60:XX), good morphology and normal growth (Fig. 1) that was used later on for NT experiments and confirmed the normal count for non-transgenic cells (data not shown).

Oocyte collection, maturation and nuclear transfer

Bovine ovaries were collected at a local slaughterhouse (Frigorifico Temuco) and grade 1 cumulus oocyte complexes (COCs) were aspirated from 2-7 mm follicles using an 18-gauge needle connected to a vacuum pump set to 60-70 mm Hg. *In vitro* maturation was carried out in TCM-199 (Sigma-Aldrich, St. Louis, MO, USA) medium supplemented with 10% inactivated FBS, 6 µg/ml luteinizing hormone (LH; Sioux Biochemical, Inc., Sioux City, IA, USA), 6 µg/ml follicle stimulating hormone (FSH; Sioux Biochemical, Inc., Sioux City, IA, USA) and 1 µg/ml Estradiol (Sigma-Aldrich, St. Louis, MO, USA) at 38.5ºC in 5% CO_2 and saturation humidity. After 17 h of maturation, oocytes were stained with Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) and enucleated by aspiration of the MII plate using an inverted microscope (Nikon TS100, Nikon Instruments Inc., Melville, NY, USA) and Narishige micromanipulators. Prior to nuclear transfer, transfected and non-transfected donor cells (passage number 5-7 and 4-6, respectively) were grown to confluency for 5 days in order to induce quiescence. These cells were microsurgically placed into the perivitelline space evacuated during enucleation and cell-cytoplast complexes were fused in sorbitol media

with a single DC pulse of 180 volts/mm and 15 μseconds delivered by an Electrocell Manipulator 830 (BTX, Harvard Apparatus, Inc., Holliston, MA, USA). Activation was carried out with 5 μM Ionomicin for 5 min followed by incubation in KSOM 0.4% BSA medium (EmbryoMax®, Millipore Corp., Billerica, MA, USA) containing 2 mM DMAP for 4 h. After activation, NT units were cultured in 50 μl drops of KSOM 0.4% BSA medium at 38.5°C with a gas mixture of 5% CO_2, 5% O_2 and 90% N_2. On day 3 (NT = day 0), cleavage rate was recorded and embryos were transferred to KSOM + 5% FBS and cultured until day 7 for embryo assessment of blastocyst rate, cell counting, RNA analysis and/or embryo transfer.

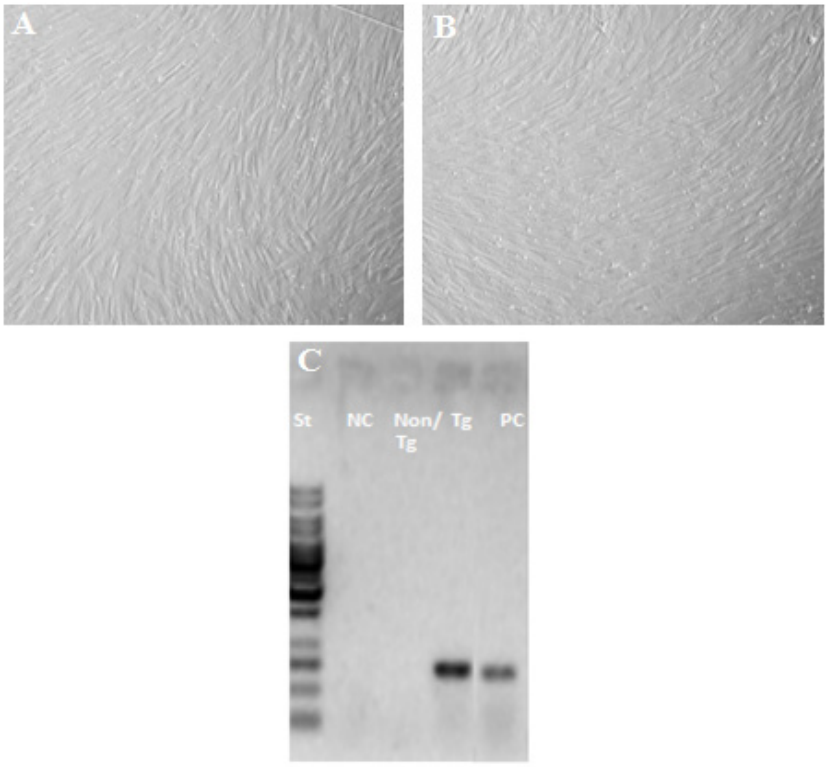

Figure 1. Morphological and molecular assessment of transfected and non-transfected cells. Panel A: Morphological assessment of non-transfected fibroblasts at confluency (passage 3). Panel B: Morphological assessment of transfected fibroblasts cell line 1 at confluency (passage 5). Magnification 100 X. Panel C: Molecular analysis for the presence of the transgene (St: Standard 100 bp Promega; NC: negative control; Non/tg: non transfected cells; Tg: Transfected cells showing a 290 bp amplified fragment of the promoter region of beta-casein; PC: Positive control for the PCR reaction).

In vitro fertilization (IVF)

Oocytes matured for 24 h (as described above) were co-incubated with sperm for 18-20 h in IVF-TL supplemented with 0.2 mM sodium pyruvate, 6 mg fatty-acid-free BSA and 0.025 mg gentamicin sulphate per ml (Parrish *et al.*, 1986). Final IVF-TALP contained PHE (80 μM penicillamine, 40 μM hypotaurine, 10 μM epinephrine), 2 μg heparin and 1 x 10^6 Percoll separated frozen-thawed sperm per ml. *In vitro* maturation and fertilization were conducted in 400 μl drops (50 COCs and/or eggs per well) at 38.5°C and 5% CO_2 in a humidified atmosphere, while embryo culture was carried out under the same conditions as described above for NT.

Cell number count

Cell number of blastomeres was assessed in good quality day 7 expanded transgenic and non-transgenic nuclear transfer blastocysts by incubating embryos (7 per treatment) in TCM-199 medium containing 10 μg/ml bisbenzamide in absolute ethanol at room temperature for 10 min. Blastocysts were then treated in 50% (v/v) glycerol, mounted onto a glass slide and visualized under an epifluorescent microscope coupled with a UV-2E/C DAPI filter.

RNA extraction, reverse transcription and gene expression analysis

Fifteen good quality expanded blastocysts divided in 3 pools per treatment (IVF and transgenic nuclear transfer embryos) were lysed in 20 μl of extraction buffer (XB; Arcturus, Carlsbad, CA, USA) by incubation at 42°C for 30 min followed by centrifugation at 3000 x g for 2 min and then stored at -80°C until used.

Total RNA was extracted from each pool of embryos and residual genomic DNA was removed by DNAse I digestion using a RNase-Free DNase Set (Qiagen, Valencia, CA, USA). Total RNA was extracted using the PicoPure RNA Isolation Kit (Arcturus, Carlsbad, CA, USA) according to the manufacturer´s instructions. Reverse transcription assay was carried out by using the RevertAid^{TM} H Minus First Strand Kit (Fermentas, Inc., Glen Burnie, MD, USA). Quantification of a panel of 20 developmentally important genes (Felmer *et al.*, 2011) was done by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) using Brilliant® II SYBR® Green QPCR Master Mix (Stratagene Agilent Technologies, Inc., La Jolla, CA, USA) in a MX3000P thermocycler (Stratagene). Dissociation curves were performed after each PCR run to ensure that a single PCR product had been amplified.

The comparative Ct method was used for quantification of mRNA expression levels using the amplification efficiency of each gene as a correction factor (Livak and Schmittgen, 2001). As reference genes, the geometric average of the *BAX*, *YWHAZ* and *HDAC2* genes were used, after being previously analysed with the geNorm Visual Basic Application Program for Microsoft Excel, as described by Vandesompele *et al*. (2002), confirming their stability under our laboratory conditions (data not shown). To measure the differences in expression, we used the pair-wise fixed reallocation randomization test in the Relative Expression Software Tool (REST; V2.0.7, Copyright 2008, Corbett Research Pty. Ltd., Munich, Germany; Pfaffl *et al.*, 2002).

Embryo transfer and diagnosis of pregnancy

Embryo transfers were carried out at the experimental Centre of the Institute for Agricultural Research (INIA, Carillanca, Chile).

Due to our interest in generating a transgenic cloned calf and the low availability of recipients, embryo transfers were carried out mainly with transgenic nuclear transfer embryos. For this procedure, two embryos (day 7 blastocysts, day 0 = fusion/activation) were loaded into 0.25 ml straws (IMV, Maple Grove, MN, USA) in Hepes Buffered HECM medium (Seshagiri and Bavister, 1989) supplemented with 20% FBS and transferred to the recipient's ipsilateral uterine horn containing a corpus luteum (CL) by a transcervical method. Pregnancy rate was established via transrectal ultrasonography 42 days after embryo transfer.

Experimental design

In vitro embryo development was assessed in nuclear transfer embryos generated with transfected and non-transfected fetal fibroblasts used as nuclear donors. Embryo quality was assessed in expanded good quality transgenic and non-transgenic blastocysts on day 7 by a staining procedure employing Hoechst. *In vivo* embryo development was assessed by the transfer of 28 transgenic and 14 non-transgenic nuclear transfer embryos into the uterine horn of 14 and 7 recipient cows on day 7 after estrus, respectively. Finally, to evaluate the gene expression pattern of transgenic nuclear transfer embryos, RNA from 3 pools of 5 good quality expanded blastocysts was collected on day 7 of culture and compared to the gene expression pattern of a similar pool of blastocysts generated by IVF.

Statistical analysis

Data analysis for embryo development and cell count was carried out by descriptive statistics based on average and standard error calculated for each of the variables using Stat graphics Plus 5.1 Software. Student's T test was used to analyse for statistically significant differences among groups for cleavage, blastocyst rate and cell counting. For qualitative data (pregnancy rate), a Chi-square test was used. An error probability of $P < 0.05$ was considered significant.

Results

In vitro developmental potential and quality of transgenic and non-transgenic nuclear transfer embryos

The suitability of donor cells derived from transgenic and non-transgenic fetal fibroblasts for the *in vitro* developmental potential of nuclear transfer embryos was investigated. As shown in Table 1, we observed no differences ($P > 0.05$) in cleavage rates in embryos reconstructed with both transgenic and non-transgenic fetal fibroblasts (65 and 69%, respectively). However, a difference ($P < 0.01$) was observed in the rate of blastocysts on day 7 (Table 1). A higher rate of blastocysts was observed in embryos generated by non-transgenic (24%) compared to transgenic cells (13%). The quality of nuclear transfer embryos as measured by the total number of cells and by assessing some morphology aspects of their appearance was not different ($P > 0.05$) between transgenic and non-transgenic groups (Table 1).

Table 1. *In vitro* development and quality of embryos reconstructed with transgenic and non-transgenic nuclear donor cells.

Embryo status	Oocytes used	Cleavage n (%)	Blastocysts n (%)	N° of cells (x ± S.E.)
Non-transgenic	257	178 (69)	61 (24)[a]	122 ±3.2
Transgenic	249	161 (65)	32 (13)	116 ±5.0

Cleavage and blastocyst rates (6 replicates) were registered at 72 and 168 h, respectively. Data followed by different letters in the same column are significantly different ($P < 0.01$).

In vivo developmental potential of nuclear transfer embryos

In an attempt to generate transgenic cattle, a higher number of transgenic nuclear transfer blastocysts (n = 28) were transferred into 14 recipient cows (2 embryos/female), whereas non-transgenic nuclear transfer embryos (n = 14) were transferred into 7 recipient cows (2 embryos/female). Only 1 pregnancy (7%) was confirmed on day 35 in the transgenic group, while 3 pregnancies were recorded (43%) in the non-transgenic group. In this group, two pregnancies were confirmed on day 120, however, these did not continue to term and the fetuses were aborted between days 120-160. On the other hand, no pregnancies were observed beyond day 35 in the transgenic group (Table 2).

Table 2. *In vivo* development of transgenic and non-transgenic nuclear transfer embryos.

Embryo status	Recipients (n)	Blastocysts transferred (n)	Pregnant recipients on day 42 n (%)	Pregnant recipients beyond day 120 n (%)	Calves born n (%)
Transgenic	14	28	1 (7)[a]	0 (0)[a]	0
Non-transgenic	7	14	3 (43)[b]	2 (29)[b]	0

Embryo transfer was carried out with expanded blastocysts after 168 h of culture. Data followed by different letters in the same column were significantly different (P < 0.05).

Gene expression analysis

Gene expression analysis carried out on day 7 expanded blastocysts showed a decrease in the expression levels of *IFN-τ* (P < 0.05) and *HDAC1* (P < 0.05) in transgenic nuclear transfer embryos relative to embryos generated by IVF, while no differences (P > 0.05) were observed in the expression levels of 18 other genes relevant for embryo development included in the analysis (Table 3).

Table 3. Reverse transcription quantitative real-time PCR analysis in day 7 expanded blastocysts.

	Relative expression results (no. of iterations: 8.000)			
Gene	Expression	95% C.I.	P(H1)	Results
BAX	0.983			
SOD2	1.057	0.131 - 29.162	0.923	
OCT4	0.923	0.486 - 1.555	0.865	
DNMT1	0.229	0.114 - 0.404	0.067	
FGF4	1.172	0.299 - 6.076	0.832	
GLUT1	0.731	0.169 - 3.380	0.666	
IGF2	0.102	0.010 - 1.531	0.168	
IFN-τ	0.156	0.054 - 0.314	0.033	DOWN
LIF	1.168	0.539 - 2.016	0.598	
GAPDH	0.616	0.247 - 1.875	0.314	
YWHAZ	1.176			
SDHA	1.178	0.106 - 9.620	0.837	
HDAC1	0.427	0.159 - 0.842	0.045	DOWN
HDAC2	0.866			
HDAC3	0.523	0.247 - 1.066	0.183	
CAT	0.496	0.080 - 2.431	0.405	
PRDX6	0.178	0.035 - 0.828	0.067	
SOD1	0.586	0.070 - 8.705	0.57	
GPX1	0.146	0.027 - 1.508	0.133	
GSS	0.11	0.064 - 0.234	0.056	

Gene expression data analysis carried out by comparing transgenic nuclear transfer embryos relative to their IVF counterparts. Reference genes (*BAX, YWHAZ and HDAC2*) were selected based on pairwise analysis of their expression stability by the GeNorm program. *Expression*: Expression ratios obtained by using randomization and boot strapping techniques included in the REST program; 95% C.I.: Range of confidence interval (95%) for the expression ratios; *P(H1)*: Represents the probability of the alternate hypothesis that the difference between the sample and control groups is due only to chance.

Discussion

In this report, we investigated the suitability of transgenic and non-transgenic fetal fibroblasts derived from the same source for the *in vitro* and *in vivo* developmental potential of bovine nuclear transfer embryos. Fetal fibroblasts were chosen as donor cells because of their rapid growth and potential for multiple cell divisions, which make them more robust for transfection experiments (Schnieke *et al.*, 1997; Cibelli *et al.*, 1998). We observed significant differences in the *in vitro* developmental potential of nuclear transfer embryos derived from transgenic and non-transgenic donor cells, despite the fact that both donor cells originated from the same fetal source. Embryos generated from non-transgenic fetal fibroblasts showed a higher developmental rate to the blastocyst stage and higher post-implantation development compared to embryos derived from transgenic donor cells, although the quality of the embryos as assessed by morphological appearance and the total number of cells was not different.

Previous studies have shown no differences in developmental rates *in vitro* when non-transgenic adult and fetal fibroblasts of the same genotype were used for generating bovine nuclear transfer embryos (Hill *et al.*, 2000; Arat *et al.*, 2002). No differences in fusion, cleavage, blastocyst or pregnancy rates between bovine transgenic and non-transgenic ear fibroblasts were also observed by Iguma *et al*. (2005) and Zhang *et al*. (2010) with transgenic and non-transgenic goat fetal fibroblasts. However, contradictory results have been observed in other studies. For instance, when transgenic fetal and adult fibroblasts were compared to their non-transgenic counterparts, significant differences were observed in the rate of blastocysts, with embryos derived from non-transgenic cells showing a higher developmental rate, irrespective of the origin of the donor cell (fetal or adult; Arat *et al.*, 2002). A similar result was observed by Bhuiyan *et al*. (2004), who found significant differences in the efficiency in generating nuclear transfer embryos when transgenic ear fibroblasts were used to compare to their non-transgenic counterpart. In our study, we also observed a lower *in vitro* and *in vivo* developmental potential when transgenic fetal fibroblast were compared to their non-transgenic counterpart, despite the fact that transgenic nuclear donor cells had normal chromosome counts, which would indicate the competence of these cells for generating a transgenic calf. These differences in nuclear transfer efficiency between transgenic and non-transgenic cells have also been observed in other species. In the mouse, using transgenic and non-transgenic Sertoli cells, live offspring were only obtained with non-transgenic cells (Ogura *et al.*, 2000). Additionally in pigs, not only different donor cells, but also different clonal cells of transgenic fibroblasts derived from the same fetus resulted in different embryo development when used for nuclear transfer (Kuhholzer *et al.*, 2001).

Contradictory results between these studies could be attributed to different factors, including differences in the culture conditions of nuclear donor cells (Wells *et al.*, 2003) and differences in transfection protocols (plasmid, transgene, selection marker and transgene site of integration) of nuclear donor cells (Hodges and Stice, 2003; Bhuiyan *et al.*, 2004). In fact, a recent study with transfected cells harbouring different foreign genes (*hFIX*, *hALB* and *hTF*) confirmed this observation, since significant differences in fusion, cleavage and/or blastocyst rates were observed when these cells were used in nuclear transfer experiments (Fu *et al.*, 2008). In our case, an additional variation could be attributed to the difference in passage number of both donor cells at the moment the nuclear transfer experiments were carried out (4-6 for non-transgenic and 5-7 for transgenic cells, respectively), although some studies have described a better developmental rate when cells with higher passage numbers are used as nuclear donors (Kubota *et al.*, 2000).

Clearly, variations observed when transgenic cells are used as nuclear donors, as described here and elsewhere, are probably due to deviations in the epigenetic regulation of the gene expression profile of the donor cells, as an outcome of genetic disturbances resulting from manipulation and/or culture conditions. In fact, abnormalities in the gene expression profile of embryos and cloned animals have been described, which suggests that transcriptional regulation mechanisms are impaired at different levels during the reprogramming process (Dean *et al.*, 2001; Beyhan *et al.*, 2007; Suzuki *et al.*, 2008). Consequently, we sought to analyze the gene expression pattern of 20 developmentally important genes in transgenic nuclear transfer embryos in an attempt to assess if the low developmental potential observed with these embryos could be attributed to an aberrant gene expression of some of these genes.

Analysis of gene expression allowed us to confirm significant differences in the expression levels of at least 2 genes (*IFN*-τ and *HDAC1*) in transgenic nuclear transfer embryos as compared to embryos generated by IVF, both showing a lower level of expression. Interferon tau (*IFN*-τ) has been identified as the major embryonic signal in maternal recognition of pregnancy in ruminants, playing a crucial role in the establishment of pregnancy due to its antiluteolitic effect, where its main function is the inhibition of the pulsatile release of prostaglandin by the uterus, thus ensuring the extension of the corpus luteum's half-life (Bazer *et al.*, 1997). Some studies have demonstrated that mRNA and protein levels are detected as early in embryonic development as the morula and blastocyst stages (Kubisch *et al.*, 1998). However, a significant increase in the expression levels are observed between days 14 and 15 of pregnancy, this period coinciding with the elongation of the embryo, where proliferation of the trophectoderm causes a substantial increase in embryo size and trophectoderm mass and sharply declines from day 21 of gestation, a period that coincides with the adhesion of the trophectoderm to the uterus (Ealy *et al.*, 2001; Ealy and Yang, 2009).

The results observed in this study differ from those described by Arnold *et al*. (2006) and Yao *et al*. (2009), who found no differences in the expression levels of *INF*-τ in bovine embryos generated by artificial insemination (AI), IVF and nuclear transfer and/or bovine embryos produced by parthenogenetic activation (PA), IVF and nuclear transfer, respectively. However, these results are consistent with a previous study by Stojkovic *et al.* (1999), who showed that the amount of *IFN*-τ secreted was different in bovine embryos produced by AI, IVF and nuclear transfer. Several factors could explain the alteration in the gene expression of *INF*-τ in transgenic nuclear transfer embryos. In fact, it has been previously shown that the levels of expression of this gene may be affected by the culture medium composition (Kubisch *et al.*, 2001; Rizos *et al.*, 2002), the sex of embryos (Kimura *et al.*, 2004) or the cellular manipulation in the cloning process (Wrenzycki *et al.*, 2001). However, although the level of expression observed in our transgenic nuclear transfer embryos was significantly lower compared to those embryos produced by IVF, this does not fully explain the low implantation rate attributed to these embryos, as some authors suggest that the expression of *INF*-τ is critical within the first 15-25 days of embryonic development, the moment at which implantation occurs in cows (Hernandez-Ledezma *et al.*, 1992; Watson *et al.*, 1992; Ealy *et al.*, 2001). Therefore, further studies are needed in order to confirm if the expression level of *INF*-τ at different time points after embryo transfer/implantation is effectively altered. If so, the assessment of the expression level of this gene at the blastocyst stage could be indicative of the developmental potential of these embryos and might be used as a genetic marker for the selection of embryos with good developmental potential and/or to confirm the selection of a suitable donor cell population for nuclear transfer (Beyhan *et al.*, 2007).

Another gene found altered in transgenic nuclear transfer embryos was *HDAC1*. This gene encodes for histone deacetylase 1, a key enzyme in the process of nuclear reprogramming, as it plays a crucial role in the regulation of transcription in cell cycle progression and development (Wee *et al.*, 2006). Chromatin is a dynamic structure that can adopt different levels of compaction, regulating access to the information contained in the DNA and thus allowing the execution of these cellular processes. One of the mechanisms that affect chromatin modelling is epigenetic modification, such as post-translational modifications of histones, including methylation, acetylation, phosphorylation and/or monoubiquitination. These modifications play a crucial role in the control of nuclear reprogramming during early events of embryogenesis (Li, 2002). Acetylation of histones by histone acetyltransferases (HATs) neutralizes the positive charges of these proteins, increasing its hydrophobicity and weakening their bonds to DNA, which causes relaxation of the chromatin structure and promotes greater transcriptional activity. This process is a reversible reaction catalyzed by histone deacetylases (HDACs). By contrast, hypoacetylated histones are correlated with low transcriptional activity and are linked to a closed chromatin structure (Rice and Allis, 2001).

Reduced *HDAC1* expression in transgenic nuclear transfer embryos, as observed in this study, would lead to hyperacetylation of histones and thus, the stimulation of the transcriptional activity of chromatin. However, in nuclear transfer embryos, precisely the opposite is necessary to reduce the transcriptional activity from the somatic genome in order to restore normal gene expression profile during embryonic development (Eckardt and McLaughlin, 2004). It had been previously established that the *HDAC* 1, 2 and 3 genes were mostly expressed during the blastocyst stage of embryonic development, similar to *HAT*1 (McGraw *et al.*, 2003). In our study, the *HDAC*2 and *HDAC*3 genes were not affected in transgenic nuclear transfer embryos compared to embryos generated by IVF, which suggests a more important role of *HDAC*1 in nuclear reprogramming. These data agree with those described recently by Nowak-Imialek *et al*. (2008), who also found no differences in the expression levels of *HDAC*2 in nuclear transfer bovine embryos, and are also in agreement with those described by Beyhan *et al*. (2007), who found a lower level of expression of *HDAC1* in transgenic nuclear transfer embryos compared to embryos produced by IVF. Interestingly, in the work of Beyhan *et al*. (2007), the authors were able to correlate the expression levels of *HDAC*1 in nuclear donor cells with different efficiencies in generating live offspring. Cells with low efficiency in generating live offspring showed reduced expression levels of *HDAC*1 at the embryonic stage of nuclear transfer embryos (morulae and blastocysts) compared to cells with high efficiency. Thus, the authors suggested that lower expression levels of *HDAC*1 could have affected the nuclear reprogramming of those cells and the final efficiency of nuclear transfer embryos (Beyhan *et al.*, 2007).

In conclusion, despite our findings, it is important to stress that other genes essential for embryonic development, which for technical reasons were not included in this study, could also be altered in these embryos. Thus, future experiments should include the analysis of other genes important for differentiation and implantation and should also extend the analysis to different stages post-implantation in order to better understand the multifactorial phenomenon of the low pregnancy rate observed particularly with transgenic nuclear transfer embryos and provide a molecular basis for new strategies to improve the efficiency of this technology in mammals.

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