Characterization of allele-specific expression of the X-linked gene *MAO-A* in trophectoderm cells of bovine embryos produced by somatic cell nuclear transfer

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ABSTRACT. Somatic cell nuclear transfer (SCNT) may affect epigenetic mechanisms and alter the expression of genes related to embryo development and X chromosome inactivation (XCI). We characterized allele-specific expression of the X-linked gene monoamine oxidase type A (MAO-A) in the trophectoderm (TF) of embryos produced by SCNT. Total RNA was isolated from individual biopsies (N = 25), and the allele-specific

expression assessed by reverse transcription-polymerase chain reaction-restriction fragment length polymorphism. Both paternal and maternal alleles were expressed in the trophectoderm. However, a higher frequency of the mono-allelic expression of a specific allele was observed (N = 17; 68%), with the remaining samples showing the presence of mRNA from both alleles (N = 8; 32%). Considering that *MAO-A* is subject to XCI in bovine, our results suggest that SCNT may influence XCI because neither an imprinted (mono-allelic expression in all samples) nor a random (presence of mRNA from both alleles in all samples) pattern of XCI was observed in TF. Due to the importance of XCI in mammalian embryo development and its sensitivity to *in vitro* conditions, X-linked genes subject to XCI are candidates for use in the development of embryo quality molecular markers for assisted reproduction.

Key words: Cloning; Trophectoderm; MAO-A; X chromosome inactivation

INTRODUCTION

Somatic cell nuclear transfer (SCNT) is a powerful reproductive technique for animal cloning and transgenic animal production, and has great potential for regenerative medicine and stem cell applications. Despite its widespread use in domestic animals, the efficiency of SCNT remains low. Most cloned embryos are eliminated *in utero,* and the few that develop to term show a high incidence of abnormalities, for example, changes in the X chromosome inactivation (XCI) process (Xue et al., 2002; Nolen et al., 2005; Oikawa et al., 2014), and placentation abnormalities (Young et al., 1998; Xue et al., 2002; Chavatte-Palmer et al., 2012). XCI equalizes the expression of X-linked genes between the sexes (Lyon, 1961; Heard and Disteche, 2006) through a mechanism known as dosage compensation; an aberrant establishment of this process may prevent embryo development. As XCI is one of the most important events of early mammalian embryonic development, controlled by epigenetic mechanisms, it may serve as an important molecular indicator of embryo quality.

In marsupials, XCI proceeds by imprinting, through which the paternal X (Xp) is selected for inactivation (Cooper et al., 1971). In humans, XCI occurs randomly in embryonic and extra-embryonic tissues (Moreira de Mello et al., 2010). In mice, however, XCI occurs initially through imprinting (Takagi and Sazaki, 1975), silencing Xp from the 4- to 8-cell stage, followed by random XCI in the inner cell mass (ICM) (Okamoto et al., 2004; Navarro et al., 2008, 2010). This second process is accompanied by several molecular events, including expression of the X-inactive specific transcript (Xist) and regulation by its antisense transcript, Tsix (Escamilla-Del-Arenal et al., 2011). X chromosome inactivation occurs earlier in mice than in rabbits and humans (Okamoto et al., 2011), which may be explained by an identified need for rapid pre-implantation development in this species (Okamoto et al., 2005).

Although XCI affects the majority of the X chromosome, several X-linked genes are known to escape the inactivation process (Berletch et al., 2015). These genes are actively expressed in the context of silenced chromatin (Boggs et al., 2002) and have an important role in determining sex differences (Heard and Disteche, 2006; Berletch et al., 2015). The gene monoamine oxidase type A (*MAO-A*) is located on the X chromosome, and is subject to XCI (Benjamin et al., 2000; Xue et al., 2002; Stabellini et al., 2009). Xue et al. (2002) demonstrated random mono-allelic expression of *MAO-A*, confirming that this gene is subject to XCI in cattle. After XCI establishment, therefore, each embryonic cell has only one active allele in cattle.

De La Fuente et al. (1999) and Bermejo-Alvarez et al. (2011) suggested that XCI begins around the blastocyst stage in bovine embryos. In a previous experiment conducted in our laboratory (Ferreira et al., 2010), we characterized allele-specific expression of *MAO-A* in bovine embryos produced *in vitro*. In that study, we detected the presence of *MAO-A* mRNA of maternal origin only at the morula stage, and both maternal and paternal *MAO-A* mRNA at the blastocyst stage, thus reinforcing that the blastocyst stage is an important developmental period of XCI in cattle.

Considering that XCI is an essential event ensuring normal embryo development, and that placentation abnormalities are important causes of low efficiency of SCNT (Chavatte-Palmer et al., 2012), the characterization of X-linked gene expression in the trophoblastic lineage is an important strategy for improving our understanding of SCNT procedures. Accordingly, the aim of this study was to characterize allele-specific expression of *MAO-A* in the trophectoderm of bovine embryos produced by SCNT.

MATERIAL AND METHODS

Experiments were approved by the Ethics Committee on Animal Use (ECAU-protocol No. 98/2010) at the School of Veterinary Medicine and Animal Science of Universidade Estadual Paulista "Júlio de Mesquita Filho''.

Ovary collection, *in vitro* maturation (IVM), and oocyte selection

We used ovaries from crossbred cows (*Bos taurus taurus* x *Bos taurus indicus*) obtained from a local slaughter house and transported in saline (0.9% NaCl) supplemented with penicillin G (100 IU/mL) and streptomycin sulfate (100 ng/mL) at 35°C. Cumulus oocyte complexes (COCs) were aspirated from follicles 3-8 mm in diameter. Only COCs with a homogeneous granulated cytoplasm and at least three layers of compact of cumulus cells were used in the study. Selected COCs were graded I and II and transferred in groups of 20-25 to 200 μL maturation medium, covered with silicone oil, and incubated for 22 h at 39°C and 5% CO_2 in air. Maturation medium consisted of TCM - 199 Earle's salts supplemented with 10% fetal bovine serum (FBS) (both Invitrogen, Carlsbad, CA, USA); 0.01 IU/mL follicle-stimulating hormone (Sigma, Carlsbad, CA, USA); 0.1 mg/mL glutamine, and antibiotic (amikacin; 0.075 mg/mL). After IVM for 20 h, oocytes were denuded using hyaluronidase (Sigma). Oocytes were then evaluated for the presence of the first polar body (PB) under a stereomicroscope SMZ645 (Nikon Corporation, Konan, Minato-ku, Tokyo, Japan); those with a PB were selected for nuclear transfer (NT).

Somatic cell culture

Skin fibroblasts from a female Nellore cow were maintained *in vitro* as nuclei donors for the NT procedure. The cow was approximately 30 months old and had been previously genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as heterozygous (AG) for *MAO-A*. Cells were grown in 25-cm² bottles at 39°C and 5% CO_2 in air, in Dulbecco's Minimum Eagle Medium (GIBCO, Life Technologies, Carlsbad, CA, USA) supplemented with 3.7g/L sodium bicarbonate, 110 mg/L pyruvate, 10% FBS, and antibiotics. Frozen straws of 0.25 μL containing skin fibroblasts were removed from liquid nitrogen (-196°C) and thawed in a 37°-38°C water bath, then maintained *in vitro* as donor nuclei for up to seven passages.

NT

After incubation for 30 min in synthetic oviduct fluid with amino acids, citrate and myo-inositol (SOFaaci) solution containing 0.33 mg/mL cytochalas in D and 7.5 mL Hoechst 3342 (Sigma), oocytes were separated into groups of 16 to 20. Oocytes were individually fixed on a holding pipette and positioned such that the PB would remain in the four o'clock position. The PB and a portion of adjacent cytoplasm were removed by aspiration without breaking the cytoplasmic membrane. Subsequently, through the same hole opened in the zona pellucida, a somatic cell was placed in the perivitelline space. Next, both structures were subjected to the process of electrofusion in a fountain ECM200 BTX (Genetronics Inc., San Diego, CA, USA) by generating electrical pulses with two loads of 2.1 kVA/cm for 30 µs, in a 0.28 MD-mannitol solution containing 0.1 mM $MgSO_4$ (both Sigma) in a melting chamber for 30 min. Next, the activation process was performed, in which the fused structure underwent incubation for 5 min in SOFaaci with 5 µM ionomycin (Sigma), followed by 4-h incubation in SOFaaci with 2 mM 6DMAP (Sigma).

In vitro embryo culture

Following NT, structures were co-cultured for 8 days in SOFaaci under a cumulus cell monolayer at 39°C and 5% CO_2 in air. Embryos were evaluated at 48, 168, and 192 h [days (D) 2, 7 and 8, respectively] for cleavage rate evaluation and blastocyst formation.

Micromanipulation for trophectoderm isolation

At D8, embryos were evaluated and expanded blastocysts (N = 25) biopsied to separate the trophectoderm from the ICM. Expanded blastocysts were chosen for biopsy to ensure the effective separation of trophoblast cells from the ICM, because at that developmental stage, it can be quite simple to distinguish the two tissues. Biopsies were performed manually using a micromanipulator M&M (M&M - The Micromanipulator Microscope Company, Escondido, CA, USA) and stainless steel blades at angles of 15 degrees (Bio-Cut®-Blades Feather, Feather Safety Razor Co, Chome Kita-Ku, Osaka, Japan). Embryos were micro-manipulated on a 100 x 20 mm Petri dish containing 200 µL maintenance medium consisted of TCM - 199 Hank'ssalts supplemented with 10% FBS (both Invitrogen) and antibiotic (amikacin; 0.075 mg/mL).

RNA extraction and reverse transcription

Total RNA was isolated from individual trophectoderm biopsies using the Arcturus®PicoPure RNA Isolation Kit (Life Technologies), according to the manufacturer instructions. Following RNA isolation, samples were incubated with 1 U DNAse I (Invitrogen), and reverse transcribed using 200 U of SuperScript III (200 U/mL; Invitrogen) and 0.5 µg of oligo-dT$_{12-18}$ primer (0.5 µg/µL; Invitrogen). The reverse transcription protocol reaction was carried out for 5 min at 65°C, 42°C for 52 min, then enzyme inactivation at 70°C for 15 min.

RT-PCR-RFLP for *MAO-A*

To detect and characterize allele-specific expression of *MAO-A*, cDNA from individual trophectoderm biopsies (N = 25) was used as a template for nested PCR. RT-PCR-RFLP was

performed according to Ferreira et al. (2010), with minor modifications. Briefly, 4 µL cDNA was used in the first round of amplification, then 1 µL amplicon from the first round was used as a template for the second round. Amplification reactions contained 2 µL PCR buffer; 1 mM $MgCl_2$; 200 nM of each dNTP; 5 µM of each inner/outer primer; and 1 U Platinum Taq DNA polymerase (Life Technologies), in a final volume of 20 µL. Amplification was performed using the following conditions: initial denaturing at 94°C for 3 min; 40 cycles of 94°C for 30 s, annealing at either 52°C (outer) or 56°C (inner) for 30 s and extension at 72°C for 40 s; then final extension at 72°C for 10 min. After both rounds of PCR, amplicon (13 µL) was incubated overnight at 37°C with 13 U *Rsa*I restriction enzyme (New England Biolabs, Ipswich, MA, EUA). Alleles were detected using a 3% agarose (w/v) gel stained with ethidium bromide (10 mg/mL) and photographed under UV light. The "A" allele produces a 106-bp fragment whereas the "G" allele produces two fragments of 84 and 22 bp, as described by Xue et al. (2002) and Ferreira et al. (2010). As negative controls, PCRs were performed in the absence of a cDNA template.

Data analysis

Both "A" and "G" *MAO-A* alleles were identified by analyzing amplicon sizes on an agarose gel, and descriptive analysis was performed by calculating the allele frequencies.

RESULTS

Twenty-five cloned embryos were biopsied, and *MAO-A* allele-specific expression analysis performed in all trophectoderm biopsies (Figure 1). Representative RT-PCR-RFLP patterns of trophectoderm biopsies are shown in Figure 2. The allele-specific expression frequency for *MAO-A* is shown in Table 1. The "A" allele was expressed in all biopsies, whereas the "G" allele was only expressed in eight trophectoderm biopsies (Table 1). A higher frequency of mono-allelic expression of the "A" allele was observed (N = 17; 68% of total biopsies), with a lower frequency of the biopsies showing the presence of mRNA from both the "A" and the "G" alleles (N = 8; 32%; Table 1).
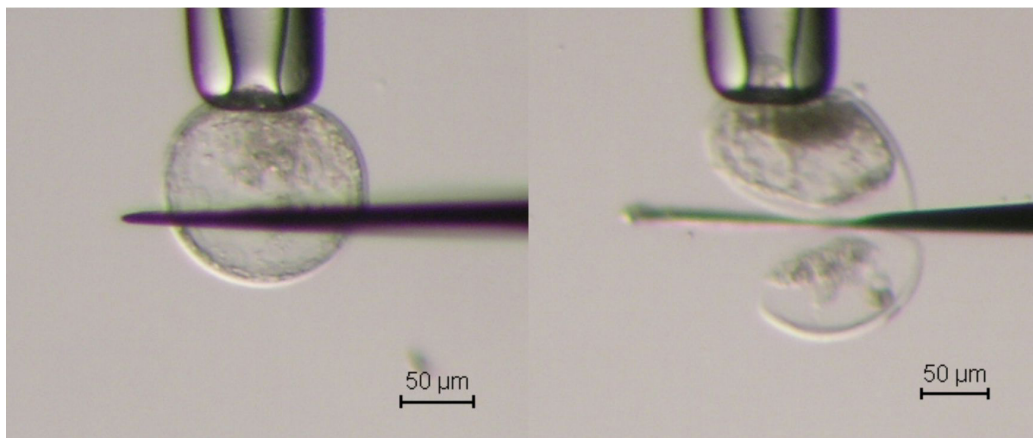
Figure 1. Biopsy procedure. An expanded blastocyst positioned to be biopsied. The trophectoderm is positioned below and the inner cell mass (darker cells) above the blade.
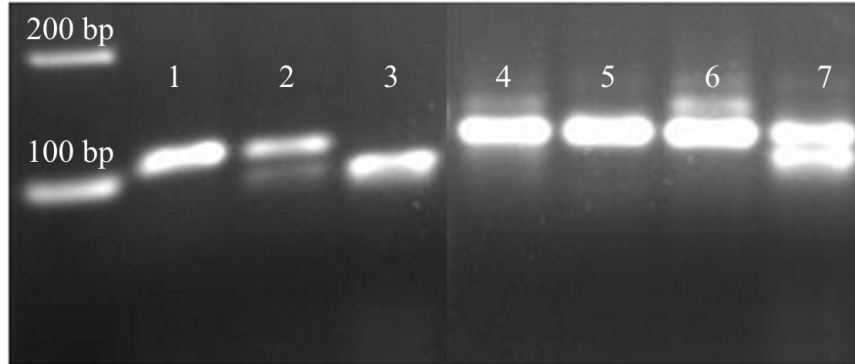
Figure 2. RT-PCR-RFLP results for *MAO-A* from individual trophectoderm samples, on 2.5% agarose (w/v) gel. Controls are genomic DNA: *lane 1* = AA; *lane 2* = AG and *lane 3* = GG. *Lanes 4*, *5*, and *6* show only the "A" allele; *lane 7* shows both "A" and "G" alleles.

Table 1. Frequency of allelic-specific expression of *MAO-A* in trophectoderm biopsies of cloned bovine embryos.

Tissue	mRNA	Embryos (N)	Frequency (%)
Trophectoderm (N = 25)	A	17	68
	G	Not detected	Not detected
	AG	8	32

DISCUSSION

We have previously described the allele-specific expression pattern in bovine embryos produced *in vitro* from the 4-cell stage to the expanded blastocyst stage (Ferreira et al., 2010) for *MAO-A*, which is subject to XCI (Benjamin et al., 2000, Xue et al., 2002; Stabellini et al., 2009). In that study, we detected only the maternal allele at the morula stage, and both alleles were detected at the expanded blastocyst stage. A similar pattern was found in mice (Okamoto and Heard, 2009).

In the present study, we characterized *MAO-A* allele-specific expression in single trophectoderm biopsies from bovine cloned embryos. The "A" allele was expressed in all biopsies, with the "G" allele expressed in only eight biopsies. A higher frequency of mono-allelic expression of the "A" allele was observed with a lower frequency of the biopsies showing the presence of mRNA from both "A" and "G" alleles.

Although cloning by SCNT is widely used, the efficiency of this technique is low (Cibelli et al., 2002). There is a consensus in the literature that aberrant epigenetic reprogramming of the somatic cell genome is the initial cause of this low efficiency (Reik et al., 2001; Blelloch et al., 2006; Eilertsen et al., 2007) and induces later placentation abnormalities, which are frequently observed in cloned animal pregnancies (Young et al., 1998; Chavatte-Palmer et al., 2012). Morgan et al. (2005) suggested that incomplete epigenetic reprogramming occurs in the majority of cloned embryos, resulting in an aberrant gene expression pattern and abnormal embryonic development.

The process of XCI, first described by Lyon (1961), is established during early mammalian embryonic development by epigenetic mechanisms such as DNA methylation and histone modification (Plath et al., 2002). Although XCI affects the majority of the X chromosome, several

genes located on this chromosome are known to escape the inactivation process (Berletch et al., 2015), unlike *MAO-A*, which is subject to XCI (Benjamin et al., 2000; Xue et al., 2002; Stabellini et al., 2009). Yang et al. (2007) commented that XCI can be aberrantly established in cloned embryos due to imprinting pattern alterations in the placenta. Therefore, characterization of the allele-specific expression patterns of genes located on the X chromosome in cloned embryos can contribute to understanding the effects of SCNT procedures on epigenetic reprogramming in embryos.

Five decades have passed since XCI was first described. Nevertheless, the role of genomic imprinting (Xue et al., 2002; Zeng and Yankowitz, 2003; Senda et al., 2004, Nolen et al., 2005; Smith et al., 2005; Ferreira et al., 2010; Bermejo-Alvarez et al., 2011) remains to be determined for different species with the exception of mice, in which the process is relatively well established (Eggan and Jaenish, 2003). Yang et al. (2007) reported that cloned embryos have a tendency for preferential X inactivation in trophectoderm cells, based on an imprinting pattern that follows the pattern of X inactivation from the donor cell genome.

We used 25 *MAO-A* heterozygous donor cells (AG genotype) to produce the embryos evaluated in this study. The adult skin fibroblasts originally came from the ICM of an embryo. XCI occurs randomly in ICMs, and once established, chromosome silencing is stable and heritable through subsequent cell divisions due to preferential inactivation (Brockdorff, 2011). Therefore, considering a preferential inactivation that follows the pattern of X inactivation from the donor cell (Yang et al., 2007), it would be expected that approximately 50% of biopsies would show "A" mono-allelic expression, and 50% would show "G" mono-allelic expression. On the other hand, if we consider that a correct event of X reactivation occurs in the earliest divisions of embryonic cells and considering a random process of X silencing, we would predict all trophectoderm samples show the presence of both alleles as we analyzed a pool of cells in each biopsy. The same pattern of *MAO-A* expression would be expected, if we consider that XCI has not yet been established at this stage of development. This would be in agreement with Bermejo-Alvarez et al. (2011), who suggested that XCI commences around the blastocyst stage in cattle. However, our results are not in agreement with either pattern. Neither an imprinted/preferential nor a random pattern of expression was found. Instead, we have detected a prevalence of mono-allelic expression favoring the "A" allele in the majority of samples. Some studies have shown aberrant expression of X-linked genes in placenta (Senda et al., 2004; Nolen et al., 2005, Yang et al., 2007), although Smith et al. (2005) found no evidence of abnormal X-linked gene expression in blastocyst-stage embryos. Bourc'his et al. (2001) commented that aberrant DNA methylation reprogramming occurs in SCNT bovine embryos. Importantly, Liu et al. (2008) showed aberrant DNA methylation patterns in four imprinted genes including *MAO-A*, in spontaneously-aborted SCNT-cloned fetuses. Dean et al. (2001) reported that epigenetic reprogramming occurs incompletely and is aberrant in most cloned embryos. Morgan et al. (2005) also reported aberrant gene expression and abnormal embryonic development in cloned embryos. Experiments conducted at later stages of development found bi-allelic expression of genes linked to the X chromosome in placenta from deceased clones. However, only one active X chromosome was found in the placenta from animals that survived to term (Xue at al., 2002). Although our results do not allow us to affirm that both X chromosomes are expressed in the same cell, we detected both *MAO-A* alleles in 32% of trophectoderm samples, supporting the possibility of an aberrant bi-allelic expression of *MAO-A* in some cells. This possibility could be confirmed by genome-wide transcriptional analysis of cloned embryos, which has revealed alterations in X-linked gene expression and in genes involved in XCI (Inoue et al., 2010), and by an aberrant DNA methylation pattern found in the *MAO-A* differentially methylated region of aborted

SCNT-cloned fetuses (Liu et al., 2008). Furthermore, Nolen et al. (2005) showed that SCNT mouse embryos could reactivate the silent X chromosome, although reactivation may be incomplete; this possibility could support our results. Based on these data, we suggest that the SCNT procedure altered the allele-specific expression pattern of *MAO-A* in trophectoderm cells. Considering this gene is subject to XCI, and based on our results and from those reported in the literature (Dean et al., 2001; Bourc'his et al., 2001; Senda et al., 2004; Nolen et al. 2005, Morgan et al., 2005, Inoue et al., 2010; Matoba et al., 2011), we speculate that the SCNT procedure may influence XCI establishment in cloned embryos.

The results presented here may contribute to a better understanding of XCI during SCNT, and of the effects of *in vitro* manipulations on gene expression and embryo quality. Due to the relevance of the XCI process in general and for correct placentation for carrying pregnancies to term, X-linked genes subject to X inactivation are important candidate genes in the development of molecular markers for embryo quality in assisted reproduction.

Conflicts of interest

The authors declare no conflict of interest.

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