Short Communication

Expression of the *CTCF* gene in bovine oocytes and preimplantation embryos

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Abstract

The CCCTC - binding factor (CTCF) is a protein involved in repression, activation, hormone-inducible gene silencing, functional reading of imprinted genes and X-chromosome inactivation. We analyzed *CTCF* gene expression in bovine peripheral blood, oocytes and in different cellular stages (2-4 cells, 8-16 cells, 16-32 cells, morulae, and blastocysts) of *in vitro* fertilized embryos. This is the first report of *CTCF* expression in oocytes and preimplantation bovine embryos and has implications for the production of embryonic stem cells and the development of novel medical technologies for humans.

Key words: CTCF, genomic imprinting, preimplantation embryos, X-inactivation.

The zinc finger protein CCCTC-binding factor (CTCF) was initially described as a "multivalent" nuclear factor, capable of specific recognition of varying DNA sites through its 11-zinc fingers (ZFs) (Ohlsson *et al.*, 2001). Although it is described as a ubiquitously expressed factor, Loukinov *et al.* (2002) showed loss of *CTCF* expression in a select population of male germinative cells. The CTCF protein is involved with repression, activation and hormone-inducible gene silencing, creating constitutive chromatin insulators or boundaries and the functional reading of the imprinted genes (Rasko *et al.*, 2001; Klenova *et al.*, 2002).

Usually, CTCF targets show rich CpG content as evidenced in the *MYC* gene and in the imprinting control region (ICR) between the *IGF2* and *H19* genes (Holmgren *et al.*, 2001). In addition, the CTCF has been reported to interact with the ICRs mapped in the region coding for human Prader-Willi/Angelman syndromes and a differentially methylated region (DMR) within the domain of reciprocally imprinted *Dlk* and *Gtl2* genes (Klenova *et al.*, 2002). It was also observed that many types of cancers (including colorectal cancer, Wilms' tumor and bladder tumors) show abnormal methylation of CTCF targets or mutations within the ZF domain of the gene (Cui *et al.*, 2001; Nakagawa *et al.*, 2001; Filippova *et al.*, 2002; Klenova *et al.*, 2002). Many tandem CTCF-binding sites also affect the methylation-dependent silencing of the X chromosome (Chao *et al.*, 2002).

Components governing cell growth, differentiation and death may be influenced by CTCF. Some authors (Rasko *et al.*, 2001; Klenova *et al.*, 2002) have reported that concurrent effects of CTCF on multiple genes (including *p19/ARF*, *p16/INK4a*, *PIM-1*, *PLK*, *BRCA1*, *TP53*, *p27*, *E2F1*, *TERT*, and *IGF2*) synergize with reduced expression of *MYC* to induce cell cycle arrest at multiple stages of the cycle. Epimutations in DMRs overlapping CTCF-binding sites have been showed in mouse preimplantation embryos cultured in different mediums (Doherty *et al.*, 2000; Khosla *et al.*, 2001). Increased methylation of *H19*DMR and decreased developmental competence of CTCF-deficient oocytes has been reported in mouse with reduced amounts of CTCF protein (Fedoriw *et al.* 2004). However, although the CTCF protein can be involved in the establishment of normal and abnormal epigenotypes in murines the functions of this protein and its expression pattern are as yet unknown in bovine embryos and oocytes.

For our study, bovine *CTCF* gene primer sequences were obtained and designed by the comparative analysis of identity among human and various mammalian and bird species using the basic local alignment search tool (BLAST) and the Gene Runner program v.3.05 (www.generunner.com). We collected peripheral blood from 10

adult animals (bulls and dams) and oocytes from the ovaries of adult dams humanely slaughtered at an abattoir in Sertãozinho in the Brazilian state of São Paulo.

Embryos were produced by *in vitro* fertilization (Meirelles *et al.*, 2001) and pools of 50 embryos from each stage of the preimplantation period were maintained in 10 µL of *RNAlater* (Ambion, USA) at -80 °C until required. Total RNA was isolated from pools of 50 embryos and from 50 oocytes using the RNeasy Mini Kit (Qiagen, USA) and from peripheral blood with Trizol (Invitrogen, Brazil), following the manufacturer's instructions in both cases. All RNA samples were digested with 1U of Dnase I (Invitrogen, Brazil) to avoid contamination with genomic DNA and the assays were repeated at least three times. The total RNA was reverse-transcribed into cDNA according to the protocol provided with the SUPERSCRIPTII RNase H⁻Reverse Transcriptase kit (Invitrogen, USA), the *CTCF* primer pairs (Invitrogen, Brazil) being 5'-ACTTACCAGA GACGCCGGGA-3' (forward) and 5'-GCTGGTGGCTG ATAGTCTGG-3' (reverse). To exclude a possible amplification of a gene with a very similar sequence, the PCR products of the *CTCF* cDNA (533 bp) correspond to a non-overlapping region with the paralogue gene *BORIS*. Each reverse-transcribed cDNA from blood, oocyte or embryo was first amplified by PCR with the β-*actin* primer pairs (Invitrogen, Brazil) 5'-CCGTCTTCCCGTCCATCG TG-3' (forward) and 5'- GGATGCCTCTCTTGCTCTG A-3' (reverse) (cDNA = 108 bp) to ensure the absence of contaminating genomic DNA. The PCR reactions were performed in a Biometra TGradient (Biometra, UK) in a final volume of 25 µL containing 3 µL of each cDNA sample, 10 mM Tris-HCl, 1.5 mM MgCl$_2$, 100 µM of each dNTP, 0.05 mM of each primer and 1 unit of Taq DNA polymerase (Gibco BRL, USA). Thermal cycle conditions for both the *CTCF* and β-*actin* primers consisted of initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, 64 °C for 2 min and 72 °C for 2 min, with a final extension at 72 °C for 10 min. The PCR products were separated on 10% (w/v) polyacrylamide gel and silver-stained and individual bands were directly sequenced using the BigDye (Applied Biosytems, USA). The final sequence was submitted to GenBank under accession number AY205566.

The isolated *CTCF* sequence showed 93% of identity with its human counterpart. We observed *CTCF* gene expression in all the bovine samples analyzed, *i.e.* peripheral blood, oocytes and embryos at different cellular stages (2-4 cells, 8-16 cells, 16-32 cells, morulae, and blastocysts) (Figure 1A). The absence of DNA contamination was demonstrated by the amplification of the β-*actin* control and the absence of the intronic sequence (Figure 1B). Despite numerous advances in the field of *in vitro* fertilization, implantation rates per embryo transferred are still low for bovines. This may reflect epigenetic disruptions in chro-
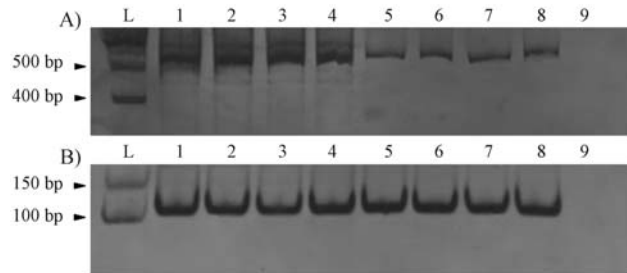
Figure 1 - A) Detection of *CTCF* transcripts: (1) immature oocytes, (2) mature oocytes, (3) 2 to 4 stage embryos, (4) 8 to 16 stage embryos, (5) 16 to 32 stage embryos, (6) morulae, (7) blastocysts and (7) blood. (L) Molecular marker, (9) Negative control. B) Detection of β-*actin* transcripts: same as 1 to 8 above.

matin structures, such as the abnormal methylation patterns observed in cloned bovine embryos and alteration of the expression of multiple developmental genes such as *IGF2* and *H19* (Dean *et al.*, 2001; Khosla *et al.*, 2001). To improve assisted reproductive techniques there is still a great need to elucidate the specific mechanisms which control the development of early mammalian embryos.

Because of the ethical issues involved with medical research involving human embryos, a good animal model is needed. There is mounting evidence that the bovine model is better than the murine model for the study of human embryonic development in respect to the timing of genome activation, intermediate metabolism and interaction with the culture medium (Wrenzycki *et al.*, 2001). However, molecular markers associated with epimutations are poorly characterized in cattle compared with mice and humans and, therefore, it is important to delineate the effective role of these genes and their expression patterns in bovine. Although *CTCF* expression has been considered to be ubiquitous cells have been detected in mouse male gonads that do not express this gene during a period that has been associated with the establishment of the imprinting marks (Loukinov *et al.* 2002).

Our observations reflect the *CTCF* transcriptional activity in preimplantation embryos and indicate the importance of such activity at this stage of mammalian development. Disruption of CTCF expression might influence the regulation of developmental genes, especially since the critical role of CTCF in the epigenetic regulation of higher order chromatin and gene silencing has been demonstrated (Schoenherr *et al.* 2003). Some candidate genes for a primary role in the development of the large offspring syndrome (LOS) phenotype in ruminants conceived by *in vitro* fertilization are regulated by CTCF (Bell *et al.*, 2000; Fitzpatrick *et al.*, 2007). Furthermore, *CTCF* down-regulation could cause loss of imprinting due to decreased rates of CTCF binding to the *H19*DMR (Fu *et al.*, 2004). This epimutation pattern is similar to those produced by approaches involving RNAi (Fedoriw *et al.*, 2004).

The causes of *CTCF* down-regulation in cell culture are as yet unknown, but the presence of fetal serum in

embryo culture mediums has been demonstrated to affect several other genes expressed during mammalian embryo development (Rizos *et al.*, 2003). Other evidence supporting CTCF involvement in epimutations is the presence of a serine-protease in fetal calf serum that cleaves CTCF protein at three sites and can result in post-translational down-regulation (Vostrov and Quitschke, 2000). Based on *CTCF* down-regulation, RNAi experiments and our results, the CTCF protein could be proposed as a candidate factor involved in the epimutations described in embryos cultured *in vitro* (Doherty *et al.*, 2000; Khosla *et al.*, 2001). Based on such evidence, we propose that a revaluation of the gene expression patterns in animals affected with LOS should focus on the *CTCF* gene and CTCF-regulated genes.

If there is the possibility that CTCF participates in the establishment of normal and abnormal epigenotypes during embryonic development, we could hypothesize a similar involvement in the development of embryonic stem cells. This should be investigated in detail because the production of embryonic stem cells is important for the development of novel medical technologies. To our knowledge, this is the first report showing *CTCF* transcripts in bovine preimplantation embryos and the presence of this gene in cattle. Our results indicate that the *CTCF* gene was not repressed during oocyte maturation during the period associated with the establishment of female imprinting in bovines, in contrast to the situation in mouse male germ cells as reported by Loukinov *et al.* (2002). This difference in gene expression pattern between male and female germ cells could be associated with the differential methylation marks on imprinted *loci*. However, future studies, including environment influences and the subsequent analysis of expression patterns during different stages of development are needed to identify the complete role and real importance of the *CTCF* gene.

Acknowledgments

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BLAST: http://www.ncbi.nlm.nih.gov/BLAST.
Gene Runner: http://www.generunner.com.

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