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Characterization of blastomeres derived from different stages of *in vitro* produced buffalo (*Bubalus bubalis*) embryos to determine the stemness/ pluripotential ability*

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Abstract

The present study was carried out to study the expression of genes for pluripotency *Oct*-4 and *Sox*-2 in blastomeres, derived from various stages of *in vitro* produced buffalo embryos, for determination of the stem cell characteristics or pluripotential ability. 0.5 per cent pronase was used for *zona lysis* and subsequent isolation of embryonic stem (ES) cells. RT-PCR was performed by using reverse specific primer (3' primer) for the first strand synthesis. Strong *Oct*-4 and *Sox*-2 expression were consistently observed in cells, obtained from 16-cell stage embryos, morula and blastocyst, whereas in cells from 2, 4 and 8-cell embryos (pre ZGA) *Oct*-4 and *Sox*-2 expression was low and variable. Out of eight trials, in three trials the blastomeres of 2-cell, 4-cell and 8-cell stage embryos (Pre ZGA) did not express *Oct*-4 gene and in five trials a faint band was observed. With regard to *Sox*-2 gene expression, in four of six trials the blastomeres of 2-cell, 4-cell and 8-cell stage embryos (Pre ZGA) a faint band was observed, but in two trials *Sox*-2 gene was not expressed, eventhough they were believed to be totipotent. Low and variable expression of *Oct*-4 and *Sox*-2 genes in early stage embryos (pre-ZGA) might be related to the exhaustion of maternally-generated *Oct*-4 and *Sox*-2 transcripts and then their recovery appears to be via expression of zygotic transcripts, which takes place in buffalo embryos from 16 cell stages onwards. Epigenetic mechanisms might be the cause of the low levels of *Oct* -4 and *Sox*-2 expression after fertilization. The sequence results for *Oct*-4 (NCBI Ac. No: EU661360) and *Sox*-2 (NCBI Ac. No: EU661361) were analyzed by BLAST and it was found that *Oct*-4 had 88% - 97% homology and *Sox*-2 - 93% to 99% homology across the phylogeny with mouse being distant relative.

Keywords: Embryonic stem cells - *Oct*-4 - *Sox*-2 - Gene expression - Buffalo

Introduction

Stem cell therapy is the latest cutting edge technology in the field of medical science for research. The embryonic stem (ES) cells among the other stem cells provide great potential to treat a vast number of diseases in animals and human beings, as they are pluripotent, meaning that they have a remarkable capability to differentiate into all cell types, including germ cells. Unlike other types of stem cells, ES cells are highly proliferative, a property that is thought to be involved in maintaining homogeneity. Somatic stem cells, such as hematopoietic stem cells, have been found to grow slowly in comparison to ES cells and they do not expand without significant accompanying differentiation (Antonchuk et al., 2002). Because of these properties, ES cells are regarded as a major potential source of material for future stem cell therapy. ES cells also provide a powerful tool for gene targeting and the generation of transgenic clonal offspring. ES cells are derived from the inner cell mass of the blastocyst-stage embryo and inner cells of post-compaction morulae, and they are capable to grow indefinitely as an established cell line. However, ES cell lines have also been derived from eight-cell (Delhaise et al., 1996) and morula-stage embryos

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(Strelchenko et al., 2004) prior to the first embryonic differentiation events, i.e., formation of the inner cell mass (ICM) and trophectoderm of the blastocyst. For establishment of bovine pluripotent stem cells, 16-cells embryos to hatched blastocyst (10-l1 days) would be suitable. However, the results in experiments for establishment of bovine embryonic stem cell lines from 2-, 4- and 8-cell stage bovine embryos haven't been consistent (Strelchenko, 1996).

The pluripotency of ES cells is maintained by a few key transcription factors, including *Oct*-4, *Sox*-2, etc. *Oct*-4 has been characterized as a transcription factor, coded by *Pou5f1* gene, which expression has been noticed to be limited in pluripotent cells of various organisms, and the zygotic expression of *Oct*-4 has been shown to be essential for establishment of pluripotent stem cell populations of the inner cell mass (Nichols *et al*., 1998). Moreover, the level of *Oct-4* has been found to govern the fate of ES cells, a critical level being required to sustain stem cell self-renewal. *Oct*-4 has also been indicated to be expressed in human ES cells, but its expression has been observed to be down-regulated in the process of their differentiation (Reubinoff et al., 2000). *Oct*-4 has been suggested to influence several genes, expressed during early embryonic development, and thus, it may be very important to the processes of development and cell differentiation (Lanza et al., 2006).

Sox-2 is a member of the *Sry* (Sex determining region-Y) related transcription factor family. Function of *Sox-2* in ES cells has first been identified in relation to *Oct*-4. *Sox*-2 and *Oct*-4 expressions overlap during early embryogenesis, and both are important for the maintenance of the pluripotent state. *Sox-2* can be regarded as one of the cofactors of *Oct-4*, since it activates the transcription of target genes, such as *Fgf-4*, *Utf-1*, *Fbx-15*, and *Lefty-1* in cooperation with *Oct-4*. Moreover, expression of *Sox-2* has been found to be regulated by *Oct-4* and *Sox-2*, indicating that a positive feedback mechanism may be involved in the maintenance of ES cell self-renewal. The role of transcription factors *Oct*-4, *Sox*-2 in ES cells has been extensively characterized in mouse and human, but in domestic animals and in particular, in buffalo (Anand et al., 2008; Kumar et al., 2008) scanty information is available and additional studies are necessary. As the stage of embryos has been established to influence the formation of embryonic stem (ES) cell lines (Ito et al., 1996), the goal of present study was to investige the expression of pluripotent genes *Oct*-4 and *Sox*-2 in stem cells derived from various stages of *in vitro* produced buffalo embryos.

Materials and Methods

The oocytes were screened under stereozoom microscope, washed thrice in 35 mm petridishes and graded based on their cumulus mass investment and homogeneity of ooplasm, as described by Nandi et al. (1998). Only 'A' and 'B' grade COCs were used for *in vitro* maturation (IVM) of oocytes in TCM-199, supplemented with 10% fetal bovine serum (FBS), 100 µM cysteamine, 1.0 µg/ml follicle stimulating hormone (FSH), 0.02 µg/ml luteinizing hormone (LH), 1 µg/ml 17-β estradiol and 50 µg/ml gentamycin. After 24 hours of maturation, oocytes with expanded cumulus mass were rinsed thrice in IVF medium (BO medium, supplemented with heparin and caffeine), and transferred to IVF droplets (10 oocytes per droplet). The 75 µl IVF droplets, containing 10 oocytes, were then inseminated with 25 µl of sperm suspension (2 x 10^6 sperm/ ml), processed by swim-up method, as described by Parrish et al. (1986), and co-incubated for 24 hours at 38.5^{0}C in 5% CO_2 in air. After 24 hours of incubation in IVF medium, oocytes were transferred to embryo-culture medium. The media, used for *in vitro* culture, were TCM-199 with BOEC clusters and mSOF. Following fertilization (day 0), oocytes were rinsed with embryo culture medium and cultured for 10 days at 38.5^{0}C in an atmosphere of 5% CO_2. During the course of culture, half of the embryo culture medium was replaced with fresh embryo culture medium on every 48 hours.

Embryos were incubated in solution, containing 0.5% pronase, until zona was removed. Embryos were observed constantly under zoom stereo microscope, until zona and trophectoderm were lysed. Residuary embryos/Zona free embryos were washed with phosphate buffered saline (PBS), containing 10% fetal bovine serum (FBS). Zona-free embryos were then incubated in Ca^{2+}/Mg^{2+} free PBS for 10 to 15 minutes at 37^{0}C in CO_2 incubator. Repeated pipetting by Pasteur-pipette disaggregated the zona-free blastomeres/inner cells.

The RNA-material was extracted from embryonic stem cells by using Trizol reagent, and One-step RT-PCR was carried out with 100ng RNA, using One-step RT-PCR kit from Invitrogen (Cat #11736-051).

Primers were designed, based on the sequences (NCBI Ac. No: DQ487023 for *Oct*-4 and DQ487021.1 for *Sox*-2) of Chinese swamp buffalos, available in Pub med GenBank, by using Lasergene software DNASTAR for *Oct*-4 and web-based primer 3-designing program for *Sox*-2, respectively.

RT-PCR for *Oct*-4 was carried out by using the forward primer (5' TGCTGCAGAA GTGGGTGGAG GAAG 3') and the reverse primer (5' CCGAGCTGCT GGGCGATGTG 3') and for *Sox*-2 - by using the forward primer (5' GCCGAGTGGAAACTTTTGTC 3') and the reverse primer (5' TGCGAAGCTGTCATA GAGTTG 3'), with the following cycling profile: cDNA synthesis for 15 minutes at 50°C; initial denaturation at 95°C for 2 minutes, followed by 36 cycles of 30 sec at 94°C, 30 sec at 55°C and 45 sec at

72°C, and a final extension for 10 minutes at 72°C. The amplified product was observed as a single band of 196bp for *Oct*-4 gene and 413bp for *Sox*-2 gene. RT–PCR product was analyzed by gel electrophoresis along with a standard 100 bp ladder as marker.

The sequences of the RT-PCR products were obtained by using specific primers from DNA sequencing facility, available at the Department of Animal Biotechnology, Madras Veterinary College, Chennai. They were analyzed for phylogenic conservation and the sequence homology across the species was established. Homologies of *Oct*-4 and *Sox*-2 genes (mRNAs) were compared with reported sequences of other species, retrieved from web-page of National Centre of the Biotechnology information (www.ncbi.nih.gov) in the BLAST (Basic Local Alignment Search Tool) search mode.

Results

Strong *Oct*-4 expression was observed in blastomeres, obtained from 16-cell stage embryos, morulae and inner cell mass of blastocyst. Out of eight trials, in three trials the blastomeres of 2-cell, 4-cell and 8-cell stage embryos (Pre ZGA) did not express *Oct*-4 gene, even though they were believed to be totipotent. However, in five trials a faint band was observed. Out of six trials, in two trials the blastomeres of 2-cell, 4-cell and 8-cell stage embryos (Pre ZGA) did not express *Sox*-2 gene, whereas blastomeres, obtained from 16-cell stage embryos, morulae and inner cell mass of blastocyst consistently expressed *Sox*-2 gene. Representative photographs of gel for *Oct*-4 gene *Sox*-2 gene expression were presented on Fig. 1 and 2.

cDNA sequences of the *Oct*-4 and *Sox*-2 RT-PCR products, submitted to NCBI Pub med GenBank, were given Accession numbers: EU661360 and EU661361, respectively. These sequences were analyzed for phylogenic conservation and sequence homology across the species was established. The nucleotide sequence of Indian water buffalo (*Bubalus bubalis*) *Oct*-4 gene had 88 - 97% homology across the phylogeny, specifically, it had 97% homology with Chinese swamp buffalo, *Bos taurus*, *Bos grunniens* and pig *Oct*-4 gene, 92% homology with rat *Oct*-4 gene, 91% homology with human and dog *Oct*-4 gene, and 88% with mouse *Oct*-4 gene.

Sequence analysis of Indian water buffalo (*Bubalus bubalis*) *Sox*-2 showed that the residues are highly conserved (93-99%) throughout the phylogeny with mouse being most distantly related and Chinese swamp buffalo and bovine sequence showing 99% homology. Specifically, it had 99% homology with Chinese swamp buffalo and bovine *Sox*-2 gene, 98% homology with sheep *Sox*-2 gene, 97% homology with dog *Sox*-2 gene, 96% homology with pig *Sox*-2 gene and 95% homology with human *Sox*-2 gene, 93% with rat and mouse *Sox*-2 gene.

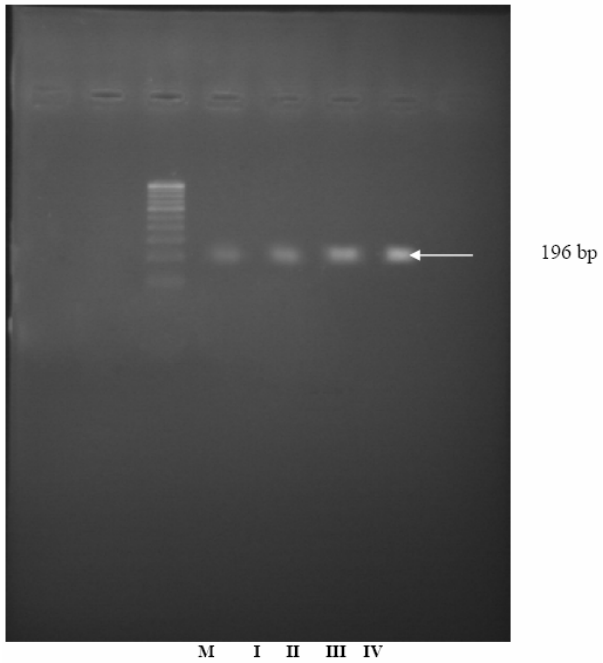

Fig. 1: *Oct*-4 gene expression in stem cells derived from different stages of embryos
I = stem cells derived from 2 and 4 cell embryos
II = stem cells derived from 8 cell embryos
III = stem cells derived from 16 cell embryos
IV = stem cells derived from morula and blastocysts
M = 100 bp DNA ladder

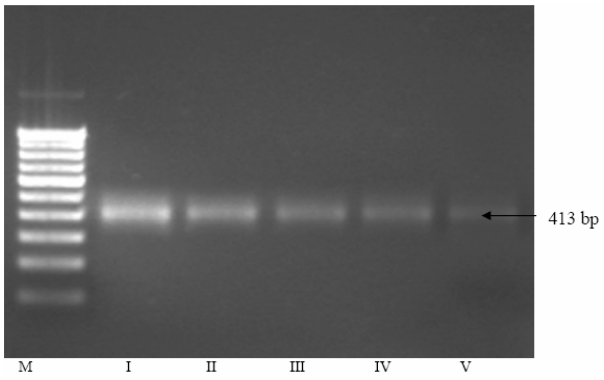

Fig.2: *Sox-2* gene expression in stem cells derived from different stages of buffalo embryos
I = stem cells derived from morula and blastocysts
II = stem cells derived from 16 cell embryos
III = stem cells derived from 8 cell embryos
III = stem cells derived from 4 cell embryos
IV = stem cells derived from 2 cell embryos
M = 100 bp DNA ladder

Discussion

POU (Pituitary Oct Unc family) domain, containing gene *Oct*-4 and HMG (High mobility group

family) domain, containing *Sox*-2 gene, have been characterized as transcription factors, essential for normal pluripotent cell development and maintenance (Avilion *et al*, 2003). Although both genes have independent roles in determining of other cell types, at least part of their function in pluripotent cells is via a synergistic interaction between them to drive transcription of target genes, such as *Fgf4, Utf1* and *Fbx15,* as well as *Sox*-2 and *Pou5f1* (gene, encoding Oct-4) themselves. Genetic link between the Sox2-Oct4 complex and *Sox*-2 and *Pou5f1* expression, as well as their *in vivo*-binding to these genes in mouse and human ESCs (Chew et al., 2005), have suggested that this complex is at the top of the pluripotent cell genetic regulatory network.

Oct-4 has been proposed as a transcriptional regulator of genes, involved in maintaining the undifferentiated pluripotent state, and it may also prevent expression of genes, activated during cell differentiation (Rosner et al., 1990). This gene has been found to activate transcription via octamer motifs, located proximal or distal to transcriptional start sites. The POU domain of *Oct*-4 is a conserved DNA binding domain that binds as a monomer to an octamer sequence motif 5'-ATGCAAAT-3'. This *cis*-acting element is important in control of the activity of many promoters and it enhancers of house-keeping and cell type-specific genes. *Oct*-4-binding sites have been found in various genes, including *fgf-4* (fibroblast growth factor-4), *pdgf r* (platelet-derived growth factor-receptor), osteopontin, *Nanog etc* (Lanza et al., 2006).

Oct-4 transcription factor is believed to co-regulate early embryonic development of mammals due to the correlation of its presence with the maintenance of pluripotency. For this reason, it is commonly used as a marker for identification of embryonic stem (ES) cells. In the present study *Oct*-4 gene expression was found throughout the pre-implantation stages studied. Strong *Oct*-4 expression was observed in inner cells, obtained from 16-cell stage embryos, morulae and inner cell mass of blastocyst. Out of eight trials, in three trials the blastomeres of 2-cell, 4-cell and 8-cell stage embryos (Pre ZGA) did not express *Oct*-4 gene, even though they are believed to be totipotent, but in five trials a faint band was observed, which can be attributed to the low levels or exhaustion of maternal transcripts in the sample. The results of the present study are in agreement with the findings of Gandolfi et al. (1997), Kurosaka et al. (2004) and Choi et al. (2007), but they are contrary to Liu et al. (2004) and Mitalipov et al. ((2003).

Oct-4 has been implicated in the protection of cells/embryos from stress-induced apoptosis. It has also been found to be essential for anti-apoptosis of stem cells in response to stress effects that might be mediated through the STAT3/Survivin pathway (Guo et al., 2008). Thus, for successful development and to attain the pluripotential ability, early stage embryo must be able to adjust its physiology in response to maternal regulatory signals and to the changes in the micro-milieu. The embryos, which fail to acquire this 'stress tolerance', either get blocked or have very low developmental competence.

Sox-2 mRNA was detected in oocytes, as well as in embryos in different developmental stages analyzed, resembling the profile of *Oct*-4. *Sox* -2 was present as both maternal and embryonic transcripts; in particular, a statistically significant increase from the 16-cell stage, concomitant with embryo genome activation, was observed, suggesting that *Sox*-2 expression might be regulated by *Oct*-4. The results of the present study are in agreement with the findings of Brevini et al. (2008).

Sox-2 and *Oct*-4 expressions overlap during early embryogenesis, and both are important for maintenance of the pluripotent state. *Sox*-2 expression in ES cells was regulated by *Sox*-2 itself and *Oct*-4, which suggested for eventual activation of gene *Sox*-2 in primitive cells by a positive autoregulatory loop. Therefore, it was speculated that the same positive feedback loop maintained the expression of *Sox*-2 and *Oct*-4 together and that the *Sox*-2 and *Oct*-4 were coordinately regulated (Boyer et al., 2005).

Sox-2 was co-expressed with *Oct*-4 in the used ES cells. *Oct*-4 and *Sox*-2 gene expression patterns were variable in stem cells, derived from early embryos, but gradually became more regular, with 100 per cent expressing *Oct*-4 and *Sox*-2 from 16-cell stage onward. Low and variable expression of *Oct*-4 and *Sox*-2 genes in early stage embryos (pre-ZGA) might be related to the exhaustion of maternally generated *Oct*-4 and *Sox*-2 transcripts, and then strong *Oct*-4 and *Sox*-2 expression, consistently observed in cells obtained from 16-cell stage embryos, morula and blastocyst, appears to be via expression of zygotic transcripts, as Zygotic genome activation takes place in buffalo embryos from 16-cell stage onwards. Epigenetic mechanism, consisting of DNA-methylation and chromatin remodeling, might be a reason of the low levels of *Oct*-4 and *Sox*-2 gene expression after fertilization (Hattori et al., 2004).

During the initial stages of zygote formation and early cleavage divisions, there is a minimal level of transcription only, so that at the time of ovulation, the mature oocyte must contain a storage pool of proteins and/or mRNA transcripts. The cycle, during which the zygote genome activated, is the longest cycle of pre-implantation development and any delay at this time would result in a decrease in the level of mRNA below critical thresholds (Rachel and Yves, 2003). Hence, the absence or low levels of *Oct*-4 expression in blastomeres of 2-, 4- and 8-cell embryos might be due to slowly cleaved embryos, used for study of gene expression. While attempting to select only viable embryos for isolation of stem cells and for subsequent

Oct-4 and *Sox*-2 expression studies, some of these early embryos might be degenerated and have been arrested, resulting in developmental block.

The variable and low levels of *Oct*-4 expression in stem cells, derived from early stages of *in vitro*-produced buffalo embryos, might be due to *in vitro*-culture, as the isolated ES-cells are from *in vitro*-produced buffalo embryos capacity to retain *Oct*-4 expression in stem cells might be lower than in stem cells, derived from *in vivo*-embryos (Tielens et al., 2006).

Critical amount of *Oct*-4 has recently been reported to be crucial for the maintenance of ES cell self-renewal. A 50% decrease or increase in the endogenous *Oct*-4 levels, relative to that of undifferentiated ES cells, resulted in differentiation of ES cells. Less subtle changes in Oct-4 level (both increase and decrease) didn't affect ES cell self-renewal. In conclusion, the precise level of Oct-4 protein governs commitment of embryonic cells along three distinct lineages (Niwa et al., 2000; Lanza et al., 2006).

The observed lack or low expression of *Oct*-4 in cells, derived from early embryos (pre-ZGA), suggested inadequate ability of those cells to retain the property of stemness/pluripotency to form primary stem cell colony and subsequently ES-cell lines, compared to inner cells derived from morulae and blastocysts (Ito et al., 1996; Hatoya et al., 2006).

Sox-2 was required by ES cells for its *Oct–Sox* enhancer activity. It has also been found to be necessary for regulation of multiple transcription factors that affect *Oct*-4 expression, and its essential function has been established to be connected with stabilization of ES cells in a pluripotent state by maintaining the requisite level of *Oct*-4 expression (Masui et al., 2007).

On the basis of the results obtained, it was concluded that *Sox*-2 is co-expressed with *Oct*-4 in ES cells and it acts synergistically with *Oct*-4 for activation of *Oct–Sox* enhancers, to regulate the expression of pluripotent stem cell-specific genes. Based on the results, it was also speculated that the observed lack or low expression of *Oct*-4 and *Sox*-2 in cells, derived from early embryos (pre-ZGA) might be a reason for inadequate ability of those cells to retain the property of stemness to form primary stem cell colony and subsequently ES-cell lines compared to inner cells derived from morulae and blastocysts, as *Oct*-4 is essential for anti-apoptosis of stem cells in response to stress effects that might be mediated through the STAT3/Survivin pathway and *Sox*-2, being responsible for maintaining the requisite level of *Oct*-4 expression.

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