SOX-2 GENE EXPRESSION PATTERN IN STEM CELLS DERIVED FROM DIFFERENT STAGES OF *IN VITRO* PRODUCED BUFFALO (*Bubalus bubalis*) EMBRYOS

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ABSTRACT

This study aims to compare the *Sox*-2 gene expression in stem cells derived from various stages of *in vitro* produced buffalo embryos. Primers were designed based on the *Sox*-2 sequence (NCBI Ac. No: DQ487021.1) of Chinese swamp buffalo available in Pubmed GenBank by a Web-based primer3 designing programme to obtain a product of 413bp. For zonalysis and subsequent isolation of ES cells 0.5 percent pronase was used. The DNA sequence of the RT-PCR product submitted to NCBI Pubmed GenBank was given accession number: EU661361. Strong *Sox*-2 expression was observed in the inner cells obtained from 16-cell stage embryos, morulae and inner cell masses of blastocyst. Out of six trials, in two trials the blastomeres/ inner cells of 2-cell, 4-cell and 8-cell stage embryos did not express *Sox*-2 gene even though they were believed to be totipotent. But in four trials a faint band was observed. The *Sox*-2 gene expression pattern was low and variable in stem cells derived from early embryos but gradually became more regular, with 100 percent expressing *Sox*-2 from the 16-cell stage onward. This might be related to the exhaustion of maternally generated *Sox*-2 transcripts and then its recovery via expression of zygotic transcripts, which takes place in buffalo embryos at the 8-16 cell stage. Epigenetic mechanisms might be the cause of the low levels of *Sox*-2 gene expression after fertilization. Based on the results it was believed that *Sox*-2 was co-expressed with *Oct*-4 in the ES cells and acts synergistically with *Oct*-4 to activate *Oct-Sox* enhancers, which regulate the expression of pluripotent stem cell-specific genes, including *Nanog*, *Oct*-4 and *Sox*-2 itself.

Keywords: embryonic stem cells, *Sox*-2, Gene expression, buffalo, *Bubalus bubalis*

INTRODUCTION

The pluripotency of ES cells is thought to be maintained by a few key transcription factors, including *Oct*-4, *Sox*-2 etc. These transcription factors in ES cells have been extensively characterized in mouse and human, but for those of domestic animals and particularly buffalo (Anand *et al*., 2008; Kumar *et al*., 2008) little information is available.

Sox-2 is a member of the Sry (Sex determining region-Y) related transcription factor family. *Sox-2* function in ES cells was first 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

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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, *Sox-2* expression is regulated by *Oct-4* and *Sox-2*, indicating that a positive feedback mechanism may be involved in the maintenance of ES cell self-renewal.

MATERIALS AND METHODS

Embryos were incubated in solution containing 0.5 percent pronase until the zona was removed. To isolate the inner cell mass (ICM), blastocysts were incubated for 3-4 minutes in solution containing calcium ionophore until the zona and trophectoderm were lysed. Embryos were observed constantly under a zoom stereo microscope until the zona and trophectoderm were lysed. Residuary embryos/ zonafree embryos were washed with phosphate buffered saline containing 10 percent FBS. Zonafree embryos were incubated in Ca^{++}/ Mg^{++} free PBS for 10 to 15 minutes at 37°C in CO_2 incubator. Repeated pipetting through Pasteur pipette disaggregates the zonafree blastomeres/ inner cells.

Sox-2 was detected by reverse transcription-polymerase chain reaction (RT-PCR). Primers were designed based on the sequence (NCBI Ac. No: DQ487021.1) of Chinese swamp buffalo available in Pubmed GenBank by using a Web-based primer 3 designing programme. RT-PCR for *Sox*-2 was carried out with 100 ng of RNA by using the forward primer (5' GCCGAGTGGAAACTTTTGTC3')andthereverse primer (5' TGCGAAGCTGTCATAGAGTTG 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 seconds at 94°C, 30 seconds at 55°C and 45 seconds at 72°C, and a final extension for 10 minutes at 72°C. These primers amplified a fragment of 413 bp. The *Sox*-2 RT-PCR product was analyzed by gel electrophoresis along with a standard 100 bp ladder as marker. Then the gel was visualized under a UV light gel documentation unit.

The cDNA sequence of the *Sox*-2 RT-PCR product submitted to NCBI Pubmed GenBank was given accession number: EU661361.The sequences were analyzed for phylogenic conservation and the sequence homology across the species was established. Homologies of the *Sox*-2 gene (mRNAs) were compared with reported sequences of other species retrieved from annotated databases such as the National Centre of the Biotechnology information (www.ncbi.nih.gov). The BLASTn search of highly similar sequence homology were explored. The cDNA sequences of the *Sox*-2 gene were phylogenetically analysed using Lasergene version 4.1 (DNASTAR Package, USA). The *Sox*-2 gene sequences used for comparison are presented in Table 1.

RESULTS AND DISCUSSION

I In two out of six trials the blastomeres/ inner cells of 2-cell, 4-cell and 8-cell stage embryos (Pre ZGA) did not express the *Sox*-2 gene whereas inner cells obtained from 16-cell stage embryos, morulae and inner cell masses of blastocyst consistently expressed the *Sox*-2 gene. Representative photographs of gel for *Sox*-2 gene expression are presented in Figure 1.

The percentage of identity among the sequences of the *Sox*-2 gene from various isolates in comparison with MVC, TANUVAS is shown in Figure 2. The phylogenetic analysis of the *Sox*-2 gene from various species by clustralW method with

Table 1. S*ox*-2 gene sequences used for comparison of homology.

Species	Accession No	Percent homology
Bubalus bubalis (Chinese swamp buffalo)	DQ487021.1	99
Bos taurus	NM 001105463.1	99
Ovis aries	X96997.1	98
Canis familiaris	XM 545216.2	97
Sus scrofa	EU503117.1	96
Homo sapiens	BC013923.2	95
Homo sapiens	Z31560.1	95
Mus musculus	NM 011443.3	93
Rattus norvegicus	NM 001109181.1	93
Gallus gallus	D50603.1	84

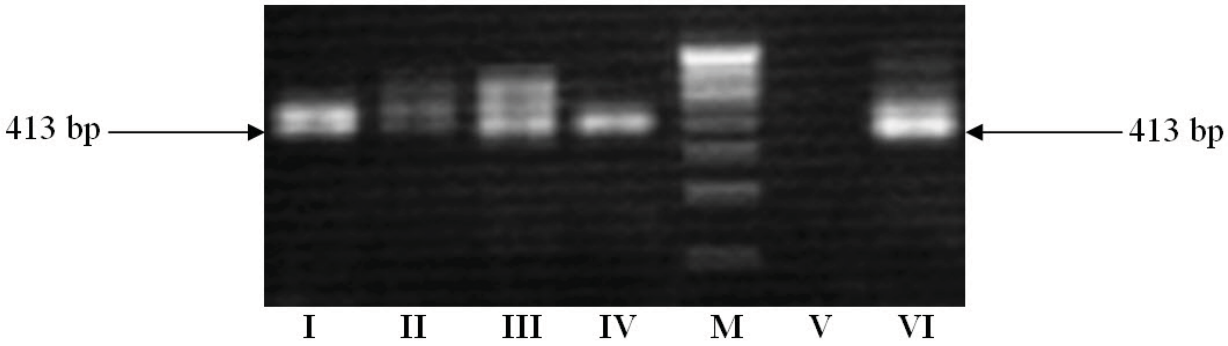

Figure 1. *Sox-2* gene expression in stem cells derived from different stages of *in-vitro* produced buffalo embryos.

Lane I = *Sox-2* amplicon in Two celled embryos
Lane II = *Sox-2* amplicon in Four celled embryos
Lane III = *Sox-2* amplicon in Eight celled embryos
Lane IV = *Sox-2* amplicon in Sixteen celled embryos
Lane M = 100 bp DNA ladder
Lane V = Negative Control
Lane VI = *Sox-2* amplicon in Morulae

bootstrap analysis, presented in Figure 2, revealed two major clusters. The *Sox*-2 gene lineage of Chinese swamp buffalo, cattle, pig, amur tiger, rhesus monkey, Norway rat, dog and lizard were grouped to the first cluster. The lineage of this first cluster again sub-grouped into three clusters of which dog, mouse, human (US, France, Yugoslavia), Norway rat, rhesus monkey, sheep and pig (USA) were grouped to one cluster, Indian water buffalo of TANUVAS, platypus and xenopus were grouped to another cluster and lizard, amur tiger, cattle (Italy), Chinese swamp buffalo and pig (China) were grouped to another cluster. The second cluster revealed a lineage covering the human (Italy), zebra fish and chicken. The bootstrap analysis is shown on every node from common ancestor.

Amino acid analysis of the *Sox*-2 gene revealed hypervaraible regions at the positions 2368-2373 and 2380. The variations were in relation with species specificity.

The HMG domain containing *Sox*-2 and the POU domain-containing *Oct*-4 were the transcription factors known to be essential for normal pluripotent cell development and maintenance (Avilion *et al.*, 2003). Their function in pluripotent cells is via a synergistic interaction between the two to drive transcription of target genes. *Sox*-2 is co-expressed with *Oct*-4 in the ES cells and acts synergistically with *Oct*-4 to activate *Oct-Sox* enhancers, to regulate the expression of pluripotent stem cell-specific genes, including *Nanog*, *Oct*-4 and *Sox*-2 itself as well as *Fgf4, Utf1,* and *Fbx15*. Each of these target genes has a composite element containing an octamer and a *Sox* binding site. Genetic links 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), suggests that this complex is at the top of the pluripotent cell genetic regulatory network.

Sox-2 mRNA was detected in oocytes as well as in embryos at the different developmental stages analyzed, resembling the profile of *Oct*-4 (Srinivasa Prasad, 2008). *Sox*-2 was present as both maternal and embryonic transcript; in particular, a 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). The *Sox*-2 gene expression patterns were variable in stem cells derived from early embryos but gradually became more regular, with 100 percent expressing *Sox*-2 from the 16-cell stage onward. This might be related to the maternal-zygotic transition (activation of the embryonic genome), which takes place in buffalo embryos at the 8-16 cell stage. This might be related to the exhaustion of maternally generated *Sox*-2 transcripts and then its recovery via expression of zygotic transcripts. An epigenetic mechanism, consisting of DNA methylation and chromatin remodeling, might be the cause of the low levels of *Sox*-2 gene expression after fertilization as reported by Hattori *et al.,* 2004.

Sox-2 expression in ES cells is regulated by *Sox*-2 itself and *Oct*-4, suggesting the possibility that *Sox*-2 is activated in primitive cells by a positive autoregulatory loop. Therefore, it is 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 are regulated coordinately (Boyer *et al.,* 2005).

The essential function of *Sox*-2 was to stabilize ES cells in a pluripotent state by maintaining the requisite level of *Oct*-4 expression (Masui *et al.,* 2007). A critical amount of *Oct*-4 has recently been reported to be crucial for the maintenance

Table 2. Percentage of identity among the sequences of *sox*-2 gene from various isolates in comparison with Indian water buffalo of TANUVAS.

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25		
1		97.7	66.1	65.9	65.3	68.8	68.7	80.1	67.5	68.1	64.6	64.6	64.6	29.1	64.5	63.4	63.1	65.3	30.7	95.2	71.2	43.7	27.2	78.8	76.6	**1**	BUBALUS BUBALIS ñMVC.TANUVAS.seq
2	2.4		97.5	97.3	95.3	94.6	94.2	95.7	94.2	94.2	94.2	94.2	94.2	32.3	93.0	92.1	91.9	88.1	34.4	97.3	84.3	58.0	30.0	84.4	81.9	**2**	BUBALUS BUBALIS (CHINA).seq
3	45.4	2.5		96.6	74.3	95.8	95.4	95.9	93.7	96.7	91.5	91.1	91.6	30.3	91.4	83.0	89.0	88.2	30.2	97.6	85.6	43.3	27.7	87.5	84.3	**3**	Bos taurus (cattle).seq
4	45.7	2.7	3.5		80.7	95.7	95.3	95.9	93.8	93.2	90.3	91.1	86.6	31.0	62.1	81.2	83.6	88.1	27.2	96.7	85.8	39.2	27.6	87.0	83.8	**4**	Ovis aries (sheep).seq
5	46.8	4.9	31.4	22.3		92.6	92.2	96.9	91.1	75.3	87.2	90.6	96.5	30.1	55.6	79.9	92.5	90.3	26.7	95.5	87.0	39.7	26.6	88.4	85.8	**5**	Canis lupus familiaris (dog).seq
6	40.6	5.6	4.3	4.4	7.8		99.5	99.8	95.2	95.3	95.4	95.4	95.6	30.2	94.7	93.0	93.7	88.0	30.7	94.3	86.3	53.5	28.6	87.5	84.3	**6**	Sus scrofa (pig).seq
7	40.8	6.1	4.7	4.9	8.3	0.5		99.4	94.7	94.8	94.9	94.9	95.0	30.4	94.3	92.6	93.2	87.6	30.7	94.0	85.8	53.5	28.5	87.0	83.8	**7**	Sus scrofa (pig)-US.seq
8	23.1	4.4	4.2	4.2	3.2	0.2	0.6		95.9	95.9	95.9	95.9	95.9	31.2	94.8	94.1	93.7	90.0	33.8	94.3	86.2	55.8	29.5	84.7	82.6	**8**	Sus scrofa (pig)-china.seq
9	42.9	6.1	6.6	6.5	9.4	5.0	5.6	4.2		98.4	97.2	97.9	98.3	30.5	95.1	92.2	93.0	88.5	30.9	94.0	85.8	53.0	27.7	87.5	85.3	**9**	Homo sapiens (human)-US.seq
10	41.9	6.1	3.4	7.2	29.9	4.9	5.5	4.2	1.7		99.9	99.9	99.9	29.4	98.2	86.5	94.6	88.7	30.7	94.0	85.8	45.6	28.5	87.0	84.8	**10**	Homo sapiens (human)-FRANCE.seq
11	48.2	6.1	9.0	10.5	14.1	4.8	5.3	4.2	2.9	0.1		100.0	95.3	30.8	96.7	91.6	89.9	88.8	30.5	94.0	85.8	49.7	28.3	87.5	85.3	**11**	Homo sapiens (human)-.seq
12	48.2	6.1	9.5	9.6	10.1	4.8	5.3	4.2	2.2	0.1	0.0		96.6	30.9	97.8	93.0	91.5	88.8	30.7	94.0	85.8	51.7	28.4	87.5	85.3	**12**	Homo sapiens (human)-YOGOSLAVIA.seq
13	48.2	6.1	9.0	14.8	3.6	4.6	5.2	4.2	1.7	0.1	4.9	3.5		31.7	62.4	90.6	92.4	89.8	28.5	94.0	85.8	43.1	28.8	87.5	85.3	**13**	Homo sapiens (human)-FRANCE(2).seq
14	229.6	191.6	213.6	198.9	226.5	255.1	241.7	211.1	229.4	249.6	208.4	219.2	195.3		30.0	31.2	31.9	30.5	37.0	30.2	31.2	28.5	41.9	33.0	30.3	**14**	Homo sapiens (human)-ITALY.seq
15	48.4	7.3	9.2	52.8	67.3	5.5	6.0	5.4	5.1	1.8	3.4	2.2	52.3	207.8		63.9	59.4	88.5	27.1	92.8	85.6	37.7	27.6	85.2	82.8	**15**	Macaca mulatta (rhesus monkey).seq
16	50.5	8.3	19.3	21.7	23.4	7.3	7.8	6.2	8.2	14.9	8.9	7.4	10.1	196.1	49.3		95.9	88.7	27.5	91.6	85.1	40.5	28.0	87.5	85.3	**16**	Rattus norvegicus (Norway rat).seq
17	51.1	8.6	11.9	18.6	8.0	6.6	7.1	6.6	7.4	5.7	10.9	9.0	8.1	192.7	58.5	4.2		89.7	27.7	91.3	84.9	42.8	30.0	87.6	86.9	**17**	Mus musculus (house mouse).seq
18	47.0	13.0	12.8	13.0	10.4	13.1	13.5	10.7	12.4	12.3	12.2	12.2	10.9	216.4	12.4	12.3	11.0		32.3	89.2	86.3	57.1	28.9	86.1	83.8	**18**	Ornithorhynchus anatinus (platypus).seq
19	193.4	157.7	213.6	279.4	350.0	206.8	206.8	164.0	206.7	209.7	210.8	207.0	246.2	143.0	350.0	273.2	279.7	176.7		36.6	32.4	27.9	33.2	28.0	26.7	**19**	Gallus gallus (chicken).seq
20	4.9	2.8	2.4	3.4	4.7	6.0	6.3	6.0	6.3	6.3	6.3	6.3	6.3	217.7	7.6	9.0	9.3	11.7	140.4		83.6	62.9	29.9	84.0	82.8	**20**	Bos taurus (cattle) ITALY.seq
21	36.5	17.6	16.0	15.8	14.3	15.2	15.8	15.2	15.8	15.8	15.8	15.8	15.8	208.4	16.0	16.6	16.9	15.2	179.7	18.6		57.1	29.6	80.3	79.9	**21**	Oreochromis mossambicus (Mozambique ti
22	104.1	61.7	105.7	124.9	122.1	72.7	72.7	66.9	74.0	97.1	83.3	77.5	106.7	261.3	133.2	118.1	108.0	63.8	262.5	51.2	63.8		26.6	82.4	80.9	**22**	Xenopus tropicalis (Silurana tropicalis
23	350.0	344.7	274.7	291.1	350.0	303.0	323.3	257.2	350.0	240.0	301.1	291.7	254.3	118.7	267.2	298.5	222.6	255.0	180.6	350.0	255.7	350.0		32.0	32.5	**23**	Danio rerio (zebrafish).seq
24	25.2	17.5	13.7	14.3	12.6	13.7	14.3	17.1	13.7	14.3	13.7	13.7	13.7	176.7	16.6	13.7	13.5	15.4	272.6	18.0	23.0	20.1	184.7		91.7	**24**	Eremias brenchleyi (LIZARD).seq
25	28.1	20.8	17.7	18.3	15.8	17.6	18.3	19.8	16.4	17.0	16.4	16.4	16.4	239.1	19.5	16.4	14.4	18.2	350.0	19.6	23.5	22.1	189.5	8.9		**25**	Panthera tigris altaica (Amur tiger).se
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**		

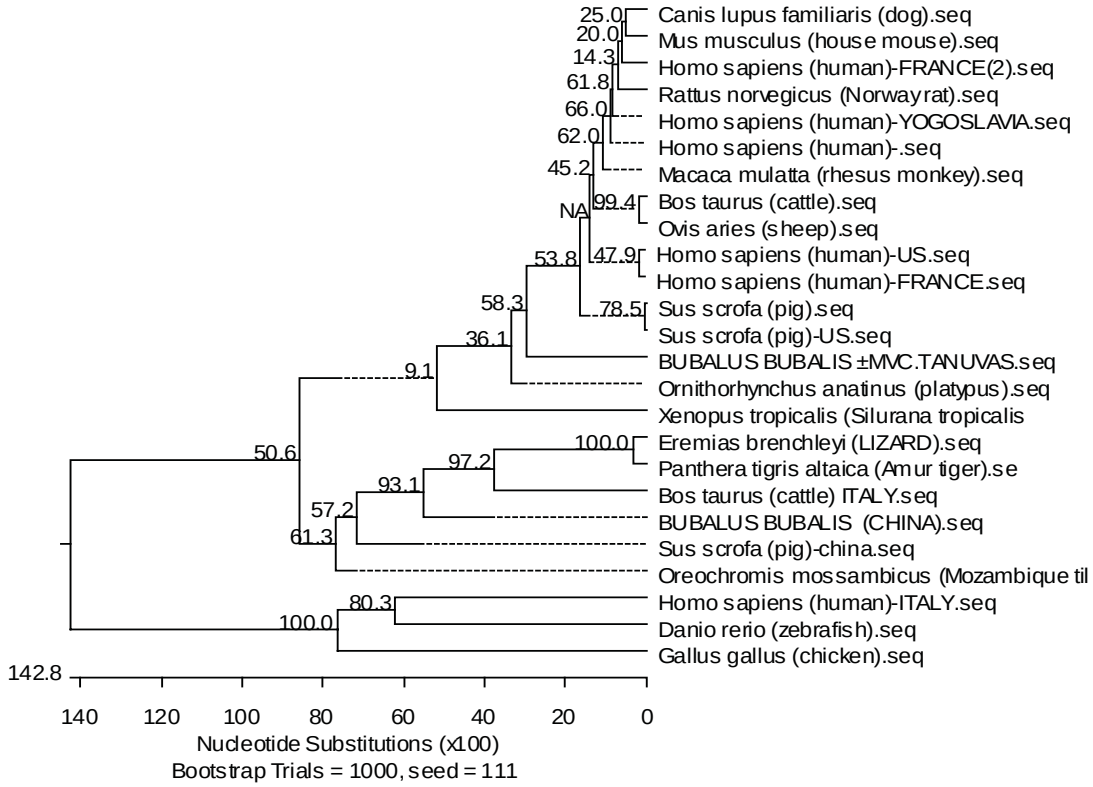

Figure 2. Phylogenetic analysis of *Sox*-2 gene from various species by ClustralW method with bootstrap analysis.

of ES cell self-renewal. A 50 percent decrease in the endogenous *Oct*-4 levels relative to that of undifferentiated ES cells results in the commitment of ES cells to trophoectoderm lineages, containing both proliferating and endoduplicating giant cells based on the culture conditions. However, an increase beyond the 50 percent threshold level of *Oct*-4 leads to the concomitant differentiation of ES cells into extra-embryonic endoderm and mesoderm. Interestingly, LIF withdrawal leads to the specification of the same lineages. Less subtle changes in *Oct*-4 level (both increase and decrease) do not 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).

Based on the results it is speculated that the observed lack or low expression of *Sox*-2 in cells derived from early embryos (pre ZGA) might be the reason for inadequate ability of those cells to retain the property of stemness, to form primary stem cell colonies and subsequently ES-cell lines compared to inner cells derived from morulae and blastocysts (Ito *et al.,* 1996; Hatoya *et al.,* 2006) as *Oct*-4 is essential for antiapoptosis of stem cells in response to stress effects that might be mediated through the STAT3/Survivin pathway, Sox-2 being responsible for maintaining the requisite level of *Oct*-4 expression (Masui *et al.,* 2007).

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