A comparative study on expression profile of developmentally important genes during pre-implantation stages in buffalo hand-made cloned embryos derived from adult fibroblasts and amniotic fluid derived stem cells

Sadeesh EM · Fozia Shah · Meena Kataria ·
P. S. Yadav

Received: 29 May 2015 / Accepted: 20 July 2015 / Published online: 30 July 2015
© Springer Science+Business Media Dordrecht 2015

Abstract Abnormal gene expression in somatic cell nuclear transfer embryos due to aberrant epigenetic modifications of the donor nucleus may account for much of the observed diminished viability and developmental abnormalities. The present study compared the developmentally important gene expression pattern at 4-cell, 8- to 16-cell, morula, and blastocyst stages of buffalo nuclear transfer (NT) embryos from adult fibroblasts (AFs) and amniotic fluid stem cells (AFSCs). In vitro fertilized embryos were used as control embryos. Alterations in the expression pattern of genes implicated in transcription and pluripotency (*OCT4*, *STAT3*, *NANOG*), DNA methylation (*DNMT1*, *DNMT3A*), histone deacetylation (*HDAC2*), growth factor signaling, and imprinting (*IGF2*, *IGF2R*), apoptosis (*BAX*, *BCL2*), oxidative stress (*MnSOD*), metabolism (*GLUT1*) regulation were observed in cloned embryos. The expression of

transcripts in AFSC-NT embryos more closely followed that of the in vitro fertilized embryos compared with AF-NT embryos. It is concluded that AFSCs with a relatively undifferentiated genome may serve as suitable donors which could be reprogrammed more efficiently to reactivate expression of early embryonic genes in buffalo NT.

Keywords Buffalo · Adult fibroblasts · Amniotic fluid stem cells · Hand-made cloning · Gene expression

Introduction

Latest studies concerning the outcome of in vitro fertilization (IVF) and somatic cell nuclear transfer (SCNT) have confirmed that embryonic developmental competence can be strictly compromised without apparent correlation with morphological changes. Superior quality embryos classified according to morphological criteria may have altered developmental capacities, with only a certain percentage of these embryos being capable of establishing pregnancy after transfer into recipients (Jousan et al. 2008). In addition, developmental competence of in vitro-produced embryos following transfer is significantly lower than that of those produced in vivo (Lonergan et al. 2003a, b). Therefore, qualitative or morphological assessment alone is not sufficient to providing an

S. EM (✉) · P. S. Yadav
Division of Animal Physiology and Reproduction, ICAR-Central Institute for Research on Buffaloes, Hisar 125001, India
e-mail: sadeeshcibr@gmail.com

F. Shah
Department of Veterinary Physiology and Biochemistry,
Lala Lajpat Rai University of Veterinary and Animal
Sciences, Hisar 125004, India

M. Kataria
Division of Biochemistry, ICAR-Indian Veterinary
Research Institute, Bareilly 243122, India

accurate and efficient estimation of embryo quality and embryonic developmental potential (Wrenzycki et al. 2004).

Gene expression analysis of pre-implantation embryonic development provides useful information to understand the changes an organism undergoes in the transition from gamete to a pluripotent embryo and into the earliest stages of cell differentiation (Steuerwald et al. 1999). Gene expression techniques have recently become a powerful tool for analyzing transcripts related to embryo quality (Warzych et al. 2007). Gene expression varies according to the respective in vitro embryo production system and nuclear transfer (NT) protocol and source of donor cells (Niemann et al. 2002; Lonergan et al. 2003a, b; Wrenzycki et al. 2004). Studies of the effects of SCNT on gene transcripts in NT-derived embryos showed that various modifications of the NT protocol (source of donor cells, passage number of donor cells, cell cycle stage of donor cells, activation protocol) have distinct effects on embryonic gene expression patterns (Wrenzycki et al. 2001; Wrenzycki and Nieman 2003). The delayed onset or absence of embryonic transcription of genes in NT embryos indicates an incomplete reprogramming of the donor nuclei with which the embryos were reconstructed, in addition, the identification of genes whose expression profiles are frequently abnormal in NT embryos will provide markers for the diagnosis of NT embryo viability prior to embryo transfer and, therefore, potentially negate the time and money-consuming transfer of non-viable embryos to recipient animals (Daniels et al. 2000). Although, the morphologic appearance of SCNT embryos does not differ from that of in vitro fertilized embryos, the potential to develop to term dramatically differs between SCNT and IVF embryos (Li et al. 2005, 2006a, b, 2008; Kato et al. 2007). Research using SCNT technology suggested that abnormal expression of genes during the earliest stages of development could be the reason of embryonic losses (Arnold et al. 2006). Abnormal gene expression might be accountable for the observed phenotypes in embryos produced by SCNT, not only during early embryonic development, but also during gestation and parturition leading in many cases to the so called abnormal offspring syndrome (AOS; Garry et al. 1996; Ranilla et al. 1998; Zhang et al. 2004). Aberrant gene reprogramming after NT has been documented also for miRNA (Castro et al. 2010). This finding

implies that de-regulated gene expression is a common phenomenon in bovine cloning. Markers that will be useful for predicting the potential of NT embryos to develop healthy offspring are needed (Kato and Tsunoda 2010).

Examination of the gene expression pattern in embryos with different developmental potential at the pre-implantation stage will be valuable, because the dynamics can be observed just after reprogramming of the donor nucleus in enucleated oocytes (Li et al. 2005). Only a very few reports are available on the comparison of gene expression in NT embryos produced from different types of somatic cells (Li et al. 2006a, b; Amarnath et al. 2007; Beyhan et al. 2007), and they were reported that there was differential expressions of genes between SCNT embryos produced from different types of donor cells. Although, no particular donor cell type has been established to be superior in overcoming the problem associated with the epigenetic reprogramming after NT and cell potential of different donors to develop normal embryos. Epigenetic markers confer stability in gene expression during mammalian development. Therefore, any changes or defects in epigenetic markers are likely to be reflected in the gene expression profile (Giraldo et al. 2007). The conventional micromanipulator based SCNT has now been replaced by hand-made cloning due to its overwhelming advantages, such as simplicity, higher efficiency, and lack of need for skilled manpower and expensive equipment (Vajta et al. 2003). It has previously been demonstrated in hand-made cloning, that the developmental potential of cloned embryos derived from pre-treatment of donor cells with oocyte extracts and selection of developmentally competent oocytes through brilliant cresyl blue (BCB) is closer to in vitro fertilized embryos in terms of developmentally important genes during maternal to zygotic transition (MZT) stages (Sadeesh et al. 2014a).

State of donor cells is one of the most important factors for cloning efficiency (Kato and Tsunoda 2010). Many attempts have been made to establish the most competent donor cell type, especially for the mouse. Compared to somatic cells, murine embryonic stem (ES) cells give higher cloning efficiency in terms of live offspring (Wakayama et al. 1999). No significant dissimilarity was observed in the cloning efficiency of mice when somatic and NT-ES cells were compared for cloning (Wakayama et al. 2005).

However, an infertile mouse was successfully cloned using NT–ES cells as donor (Mizutani et al. 2008). Although ES cells may be successful in NT in mice, this process is limited in other species where definitive ES cells have not been established so far. The ES cells competent of generating germ line chimeras have not been obtained in farm animals. The pluripotent nature of these cells is not well defined and based only on expression of pluripotency markers defined for mice and human ES cells which could be ambiguous (Munoz et al. 2008). Thus investigation of other somatic stem cells used in NT is necessary. Some researchers reported that use of undifferentiated donor nuclei is effective for generating cloned animals (Cheong et al. 1993; Hiiragi and Solter 2005). Compared to somatic cells, porcine stem cells give higher cloning efficiency in terms of in vitro and in vivo developmental ability of cloned embryos (Zhao and Zheng 2010). These results suggest that the undifferentiated state of donor cells may increase the cloning efficiency. There have been only a few published reports about the results of donor cell types on the development potential of cloned buffalo embryos, since the generation of the first cloned buffalo (Shi et al. 2007). The blastocyst formation of embryos derived from cumulus cells was higher than those of embryos derived from fetal fibroblast or adult fibroblast (Shah et al. 2009). The donor cell types, adult fibroblast, fetal fibroblast or cumulus cells had similar ability to support cleavage and embryo development (Srirattana et al. 2010). The adult fibroblast derived from ear pinna and NT–ES cell-like cells derived from cloned blastocysts generated using this adult fibroblast as donor cells, when used for NT, gave almost similar cleavage and blastocyst rates (George et al. 2011). It has been recently demonstrated an enhanced in vitro developmental potential of the cloned embryos derived from amniotic fluid stem cells (AFSCs) than that of the cloned embryos derived from adult fibroblasts (AFs) in buffalo hand-made cloning (Sadeesh et al. 2014b).

In the present study, we assessed gene expression patterns in NT embryos reconstructed with AFs and AFSCs, and compared these with that of pre-implantation in vitro fertilized embryos as control to gain more insights into the relation between the state of differentiation and cloning efficiency. Here, mainly focus is on the differences in developmentally important gene expression patterns in embryos before and after the time of the MZT period. During MZT, the

embryo undergoes several important physiological events including modification of chromatin structure (Kanka 2003) and de novo methylation of DNA (Niemann and Wrenzycki, 2000). One challenge in SCNT pre-implantation embryos is to not only initiate new gene expression at MZT, but also to turn off the expression of somatic donor cell genes at the same time (de A Camargo et al. 2005). Many of the studies related to the evaluation of differences between NT and IVF embryos reported in previous works have been carried out considering the morula and blastocyst stages, where differences in gene expression profile may be more obvious as compared to MZT stage. The gene expression analysis at these stages of pre-implantation development might offer better insight into the reprogramming process.

Materials and methods

Ethics statement, chemicals and reagents

These studies were conducted in accordance with the guidelines laid down by the CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals) and with the approval from the IAEC (Institute Animal Ethics Committee). All chemicals, reagents, culture media were of cell culture grade and obtained from Sigma Chemicals Co. (St. Louis, MO, USA) unless otherwise indicated. Fetal bovine serum (FBS) was from Hyclone (Thermo Scientific, Wilmington, DE, USA), FBS used was from the same batch throughout the study. RNase and DNase free tips, centrifuge tubes were from Invitrogen (Carlsbad, CA, USA). Disposable 35 mm × 10 mm cell culture petri dishes, four-wells multi dishes, six-well tissue culture plates were procured from Nunc (Roskilde, Denmark). Membrane filters (0.2 µm) were from Pall Life Sciences (Pall Corporation, Ann Arbor, MI, USA). The primers were synthesized by Sigma (P) Ltd. (Delhi, India).

Establishment of fibroblast cells culture

Primary ear fibroblast culture from an adult Murrah buffalo was established and prepared for cloning as reported earlier by Sadeesh et al. (2014b). Briefly, ear skin biopsies from adult Murrah buffalo were obtained aseptically in sterile phosphate buffered saline (PBS) with 1 % antibiotic–antimycotic solution and

transferred to laboratory within 10 min. Tissue samples were washed six times with Dulbecco's phosphate buffered saline (DPBS) and chopped into small pieces (0.5 mm). These were transferred to 25 cm² culture flasks and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine, 15 % FBS, 1 % non essential amino acids, 1 % vitamins and 50 µg/ml gentamicin in a CO₂ incubator (5 % CO₂ in air) at 37 °C. Cells were passaged upon reaching 70–80 % confluence by partial trypsinization with 25 % EDTA and washed with cell culture medium to remove traces of the trypsin–EDTA (up to passage 10–15). A one half portion of the cell pellet, obtained by centrifugation (200×g, 4 °C, 5 min), was re-suspended in pre-cooled (4 °C) cryopreservation medium (DMEM supplemented with 1 % non-essential amino acids, 1 % vitamins, 1 % pen/strep/amp (Gibco, Life Technologies, Grand Island, NY, USA), 10 % (v/v) dimethylsulfoxide (DMSO) and 15 % FBS) stored at –80 °C overnight, and then transferred directly to liquid nitrogen (–196 °C), whereas the rest of the cells was cultured to grow further from each passage.

Collection and transportation of amniotic fluid samples

Buffalo gravid uteri at 50–100 days gestation were obtained from slaughterhouse, washed 2–3 times with isotonic saline containing 400 IU/ml penicillin and 500 µg/ml streptomycin and transported to the laboratory in a thermally insulated ice box within 4 h. The curved crown-rump (CVR) measurement was made and fetal/gestation age was estimated using Soliman Eq. (1975), $Y = 28.66 + 4.496 X$ (If CVR is 20 cm), where Y is the age in days, and X is the CVR length in centimeters. Uterine incision, fetus and membranes were located, and amniotic fluid (AF) was aspirated aseptically with the help of a 20 ml syringe fitted with 18 gauge hypodermic needle. Amniotic fluid was collected in centrifuge tubes. The appearance and volume of fluid collected was observed.

Isolation and culture of AFSCs

The AFSCs were separated by centrifugation (400×g, 10 min) and washed twice with DPBS. The cell number was counted using hemocytometer and seeded at density of 10³ cells/cm² in 25 cm² culture flask containing cell

culture medium (DMEM supplemented with 15 % FBS, 1 % non-essential amino acids, 1 % vitamins, 1 % pen/strep/amp) and kept in humidified CO₂ incubator at 5 % CO₂ in air and at 38.5 °C. Morphological features together with the shape and size of the cells, their tendency to form aggregates and their attachment to the culture flask were recorded at an interval of 24 h. The medium was replaced every 3 days. Viability of the cells was monitored by standard protocols of exclusion of trypan blue dye and the cells were counted using a hemocytometer. The cells were allowed to grow and were subcultured by passaging after achieving 70–80 % confluence. One half portions the cells were cryopreserved from passages 8–12 in 10 % DMSO in DMEM supplemented with 30 % FBS using the same cryopreservation protocol as described for fibroblasts, whereas rests of the cells were cultured to grow further from each passage. Passage 12 was the last time point included for characterization and differentiation.

Characterization and differentiation of AFSCs

For characterization and differentiation of AFSCs the same culture protocols were used as described earlier (Sadeesh et al. 2014b).

Preparation of donor cells

Cultured AFs and AFSCs of passages 10–15 and 8–12, respectively, were used as nuclear donors. Donor cell preparations for HMC were done as described earlier (Sadeesh et al. 2014b). Briefly, after a minimum of 1 week of cryopreservation, one vial of cryopreserved cells was thawed at 37 °C in a water bath for approximately 15 s, then transferred into a centrifuge tube and the cells were suspended in the same growth medium as described for isolation and primary expansion. To remove the cryoprotectants, the contents were centrifuged twice at 200×g for 10 min, and the pellet was dissolved and cultured in a 25 cm² culture flask with culture medium for 2–3 days before use as donor cells. A small fraction of cells was used to evaluate the cell viability with trypan blue dye exclusion method and counting live (not accepting stain) and dead (stained) cells using hemocytometer under phase contrast microscope (Nikon, Tokyo, Japan). Immediately before use, the proliferated donor cells were harvested by trypsinization and washed by centrifugation and resuspended in T20 media (T

denotes HEPES modified TCM-199 supplemented with 2.0 mM L-glutamine, 0.2 mM sodium pyruvate, 50 µg/ml gentamicin and the following 20 number denotes 20 % FBS), for use as nucleus donor cells.

Collection of oocytes

Ovaries from reproductive organs of adult, apparently healthy female buffaloes collected from abattoir within 30 min of slaughter were washed three times with warm isotonic saline (35–37 °C) containing 400 IU/ml penicillin and 500 µg/ml streptomycin and transported to the laboratory within 4–6 h. Aspiration of cumulus oocyte complexes (COCs) were performed as described earlier by Sadeesh et al. (2014a). Briefly, oocytes from follicles (2–8 mm) were aspirated with 18 gauge needle attached to a 10 ml syringe (Sigma Chemical Co., # Z248029) loaded with aspiration medium (TCM-199 containing 0.3 % BSA, 0.1 mg/ml glutamine and 50 µg/ml gentamicin). The oocytes were washed four to six times with the washing medium which consisted of TCM-199 with 10 % FBS, 0.09 mg/ml sodium pyruvate, 0.1 mg/ml L-glutamine and 50 µg/ml gentamicin. The COCs having a compact and unexpanded cumulus mass with equal to or greater than three layers of cumulus cells and homogeneous granular ooplasm were selected for in vitro maturation (IVM).

In vitro maturation

The COCs were subjected to maturation in IVM medium consisting of TCM-199 + sodium pyruvate (0.80 mM) + L-glutamine (2 mM) + 10 % FBS + 5 % follicular fluid + PMSG (20 IU/ml) + hCG (10 IU/ml) + gentamicin (50 µg/ml). The pH of the medium was adjusted to 7.4 and filtered through 0.22 µm membrane filter just before use. The COCs were washed several times with IVM medium and a group of 15–20 COCs were placed independently in 100 µl droplets of IVM medium covered with sterilized mineral oil in 35 mm Petri dishes and cultured for 21 h under 5 % CO₂ at 38.5 °C.

Preparation of recipient cytoplasts and hand-made cloning (HMC)

The recipient cytoplasts preparations from in vitro matured oocytes (cumulus/zona removal and manual enucleation) and the procedures for HMC were

performed using standard protocols as described earlier (Shah et al. 2008).

Embryo culture

In vitro culture of NT embryos was performed according to the methods described previously (Sadeesh et al. 2014b). Briefly, the activated embryos were cultured in 400 µl of Research Vitro Cleave medium (K-RVCL-50, Cook, Brisbane, QLD, Australia) supplemented with 1 % fatty acid-free BSA in a four well dish (15–20 embryos/well) covered with mineral oil and kept undisturbed in a humidified CO₂ incubator at 38.5 °C. For gene expression analysis, NT embryos derived from AFs and AFSCs at 4-cell, 8- to 16-cell, morula and blastocyst stages were collected at 48 h post-activation (h.p.a), 94–96, 120–144 and 168–192 h.p.a, respectively.

In vitro embryo production through in vitro fertilization

In vitro maturation

The COCs having compact and unexpanded cumulus mass cells and homogenous granular ooplasm were subjected to IVM for 24 h in previously mentioned IVM media.

Sperm capacitation

Spermatozoa were capacitated using Brackett and Oliphant medium (BO; Brackett and Oliphant 1975). In all experiments, frozen semen from the same bull was used. Frozen semen from the buffalo bull stored in 0.25 ml straws was thawed in a water bath at 37 °C for 1 min. Spermatozoa were washed twice at 2500 rpm for 5 min using semen washing solution of BO medium supplemented with 10 µg/ml heparin, 137 µg/ml sodium pyruvate and 1.942 mg/ml caffeine sodium benzoate. The pellet was resuspended in about 0.5 ml of the washing BO medium and the sperm number was counted using hemocytometer and the number of spermatozoa were adjusted to 2×10^6 /ml.

In vitro fertilization procedure

For IVF, matured oocytes were washed twice in oocytes washing solution of BO medium and

transferred to 50 µl droplets of capacitation and fertilization BO medium (Washing medium supplemented with 10 mg/ml of fatty acid free BSA). The spermatozoa in 50 µl of the capacitation and fertilization BO medium were then added to the droplet containing oocytes, covered with sterile mineral oil and placed in CO₂ incubator at 38.5 °C for 18 h for IVF.

Embryo culture and collection

The in vitro fertilized embryos were cultured using the same culture protocol as described for the HMC embryos and for gene expression analysis, IVF embryos at 4-cell, 8- to 16-cell, morula and blastocyst stages were collected at 48 h post-insemination (h.p.i), 94–96, 120–144 and 168–192 h.p.i, respectively.

Isolation of total RNA, complementary DNA synthesis and RT-PCR analysis

Embryos were collected at different developmental stages (4-cell and 8- to 16-cell, $n = 10$, each; morula and blastocyst, $n = 5$ –6, each) from HMC and IVF were used for preparation of cDNA using cDNA kit (Ambion Inc, The RNA Company, Austin, TX, USA) according to the manufacturers' protocol. Due to transcript level variations between early- and late-cleaved embryos (Roth and Hansen 2002), only early-cleaved embryos were collected in each group. The embryos were washed with 200 µl ice cold PBS after which 50 µl of chilled cell lysis buffer was added and the mixture was incubated at 75 °C for 10 min in a thermal cycler. Genomic DNA was degraded by incubating the cell lysates in DNase-I at 37 °C for 30 min and the remaining activity of DNase-I was inactivated by heating at 75 °C for 5 min. For cDNA synthesis, 10 µl of the cell lysates (RNA), 4 µl dNTP mix (2.5 mM each dNTP), 2 µl random decamer were transferred to 200 µl PCR tube. The reaction mixture was incubated at 70 °C for 1 min to denature RNA for easier binding of primer in the thermal cycler. The tubes with the reaction mixture were immediately cooled over ice, and the remaining reverse transcriptase reagents (2 µl 10X RT buffer, 1 µl MMLV reverse transcriptase and 1 µl RNase inhibitor) were added. The reaction mixture was again mixed and incubated in a thermal cycler at 42 °C for 60 min and 95 °C for 10 min to inactivate the reverse transcriptase. The

synthesized cDNA was stored at –80 °C until used for amplification step.

Real-time PCR for relative quantification

Real-time PCR (Applied Biosystems 7500 Real-Time PCR system) was performed using SYBR green qPCR supermix (Invitrogen SYBR Green qPCR supermix: Carlsbad, CA, USA) as a double—standard DNA specific fluorescent dye in 25 µl reaction to assess the gene expression of *OCT4*, *NANOG*, *STAT3*, *DNMT1*, *DNMT3A*, *HDAC2*, *IGF2*, *IGF2R*, *BAX*, *BCL2*, *MnSOD* and *GLUT1*. Validation studies were performed using *GAPDH* as the reference control and *GAPDH* expression was consistent across various stages between embryos derived from NT and IVF. All genes of interest were analysed in triplicate with different samples. The amplification was carried out in 25 µl volume reaction mixture containing 12.5 µl of SYBR Green qPCR mastermix, 1 µl of primer (10 pM each forward and reverse primer), 2 µl of cDNA template and 9.5 µl of nuclease free water. Samples not exposed to reverse transcriptase (RT) were used as negative controls. The PCR reactions were started at 95 °C for 10 min, followed by 40 cycles of 45 s at 95 °C for DNA denaturation, 30 s at the specific primer annealing temperature (Table 1) and 30 s at 72 °C for elongation in each cycle. The comparative CT method was used for relative quantification of target gene expression levels. Quantification was normalized to the internal control *GAPDH* gene. Within long-linear phase region of the amplification curve, each cycle doubled the amplified product. The ΔCT value was determined by subtracting the *GAPDH* CT value for each sample from the target gene CT value. Calculation of $\Delta\Delta CT$ value involved using the highest sample method ΔCT as an arbitrary constant to subtract from all other ΔCT samples values. Fold changes in relative mRNA expression of the target genes were determined using the formula $2^{-\Delta\Delta CT}$.

Statistical analysis

Data were analysed using the GraphPad Prism (version 6.05). Experimental results were presented as mean $\pm$ SEM. Data were subjected to analysis of variance, and the Tukey test was used to separate the means ($P < 0.05$) that were considered statistically significant.

Table 1 Details of primer sequence for quantitative RT-PCR and qPCR

Gene name	Sequence (5'–3')		Annealing temp (°C)	GenBank accession no.
GAPDH	Forward	CCTGCCAAGTATGATGAGA	53	GU324291
	Reverse	GAAGGTAGAAGAGTGAGTGT		
OCT4	Forward	GACAAGGAGAAGCTGGAG	54	JF898834
	Reverse	GCAAATTGTTCAAGGTCTTTC		
NANOG	Forward	GGGAAGGGTAATGAGTCCAA	56	DQ487022
	Reverse	AGCCTCCCTATCCCAGAAAA		
HDAC2	Forward	GACATATGAGACTGCAGTTGC	57	XM_004370 57
	Reverse	ACCTCCTTCTCCTTCATCCTC		
DNMT1	Forward	ATAAGTAAGATAGTGTTGAGTTC	55	NM_182651
	Reverse	TTGAGCATACAAGGAGGAA		
DNMT3A	Forward	GACAAGAATGCCACCAAAGC	55	AY271298
	Reverse	ATCCACCAAGACACAATGCG		
STAT3	Forward	GCGAAGAATCAAGCAGTTCC	60	DQ487026
	Reverse	CCAGGGCAGTAAGCATCTGT		
IGF2	Forward	CTTCAGCCGACCATCCAG	55	FJ032306
	Reverse	GGGGTGGCACAGTAAGTC		
IGF2R	Forward	GCAGATTTATTTCTTCTCCAC	55	J03527
	Reverse	CACTCAAACCTCGTAGAAGCA		
MNSOD	Forward	GGGCCATATCAATCACAG	55	JN687584
	Reverse	GTCCCTGCTCCTTATTG		
GLUT-1	Forward	CCGTCTCTTCTATCCAA	56	HQ434959
	Reverse	GGTCTTCTTGAATAGTGAGTT		
BAX	Forward	ATCCACCAAGAAGCTGAG	52	HE661581
	Reverse	CTGCGATCATCCTCTGTA		
BCL2	Forward	GGCCCCTGTTTGATTCT	56	U92434
	Reverse	CTTATGGCCCAGATAGGC		

Results

Embryo production

The IVF and SCNT embryonic development is presented in Fig. 1. The cleavage rate was significantly ($P < 0.05$) higher in IVF (82.1 ± 9.2) than in NT embryos from AF and AFSC (62.8 ± 1.8 and 71.1 ± 1.2 , respectively). However, blastocyst rates in IVF and NT embryos derived from AFSCs did not differ (31.1 ± 4.7 and 29.9 ± 2.2 , respectively) but were significantly ($P < 0.05$) higher than in NT embryos from AFs (19.1 ± 1.5). Total cell number did not show significant ($P > 0.05$) differences between IVF and NT embryos derived from AFSCs (186 ± 4.2 , and 172.4 ± 5.8 , respectively) but were significantly ($P < 0.05$) higher than from AFs (148.2 ± 6.1).

Comparison of gene expression patterns in embryos derived from different origin

Expression of pluripotency and transcription related genes

The relative abundance (RA) of *OCT4* transcript at the 4-cell, 8- to 16-cell and blastocyst stages, levels did not vary between AF and AFSC-NT embryos, but were significantly ($P < 0.05$) lower than IVF embryos. The difference in *OCT4* transcript between the NT embryos was significant ($P < 0.05$) only at the morula stage (Fig. 2a). The RA of *NANOG* transcript was significantly ($P < 0.05$) higher at the 4-cell stage in AF-NT embryos, whereas no difference was observed between IVF and AFSC-NT embryos. At the 8- to 16-cell stage, levels did not vary between AF and AFSC-NT embryos, but were significantly

Fig. 1 Different development stages of buffalo after in vitro fertilization (**a–d**) and cloning (**a1–d1**) embryos. **a, a1** 4-cell stage embryo; **b, b1** 8-cell stage embryo; **c, c1** 16-cell stage embryo; **d, d1** blastocyst (50 μ m)

($P < 0.05$) higher than IVF embryos. No significant ($P > 0.05$) difference in the RA between IVF and AF-NT embryos was observed at the morula stage, while a minimal abundance was detected in AFSC-NT embryos. Differences between IVF and NT embryos were significant ($P < 0.05$) at the blastocyst stage, where the levels were higher in NT embryos (Fig. 2b). For *STAT3* transcript, at the 4-cell stage, it was significantly ($P < 0.05$) higher in IVF embryos than in NT embryos. At the 8- to 16-cell and morula stages, *STAT3* transcript level was significantly ($P < 0.05$) lower in IVF embryos than in NT embryos. Difference between IVF and NT embryos were significant ($P < 0.05$) at the blastocyst stage, where the levels were higher in AFSC-NT embryos. Significantly ($P < 0.05$) higher RA of *STAT3* was observed in AF-NT embryos at the 4-cell, 8- to 16-cell and morula stages. At the blastocyst stage, it was significantly ($P < 0.05$) higher in AFSC-NT embryos than in the AF-NT counterparts (Fig. 2c).

Expression of DNA methyltransferase and histone deacetylase-related genes

The *DNMT1* transcript level was significantly ($P < 0.05$) higher at all stages of NT as compared to IVF, except at blastocyst stage. In NT embryos it was higher at the 8-cell stage than at the morula and blastocyst stages. The *DNMT1* levels at the 4-cell, 8- to

16-cell, and morula stages were significantly ($P < 0.05$) higher in AF-NT embryos followed by AFSC-NT and IVF embryos, but there were no differences at the blastocyst stage (Fig. 2d). The RA of *DNMT3A* was high at the 4-cell stage, but decreased markedly at the 8- to 16-cell, morula, and blastocyst stages in NT embryos. Between IVF and NT embryos, except at the blastocyst stage, a significantly ($P < 0.05$) higher abundance of *DNMT3A* was observed at other developmental stages in AF-NT embryos (Fig. 2e). Levels of *HDAC2* decreased gradually from the 4-cell to morula stages in NT embryos. At the 4-cell and 8- to 16-cell stages, it was significantly ($P < 0.05$) higher than IVF embryos. At the 8- to 16-cell stage, no significant ($P < 0.05$) variation was observed between NT embryos, but the level was significantly ($P < 0.05$) higher than in IVF embryos. No significant differences ($P > 0.05$) were found between IVF and AFSC-NT embryos at the blastocyst stage, whereas significantly ($P < 0.05$) lower levels were detected in AF-NT embryos (Fig. 2f).

Expression of imprinting related genes

The *IGF2* transcript level was significantly ($P < 0.05$) higher at all stages in IVF and AF-NT embryos than in AFSC-NT embryos. Moreover, it was significantly ($P < 0.05$) higher at the 8- to 16-cell stage and further down-regulated at the blastocyst stage in NT embryos

Fig. 2 Transcript abundance of *OCT4*, *NANOG*, *STAT3*, *DNMT*, *DNMT3A*, *HDAC2*, *IGF2*, *IGF2R*, *BAX*, *BCL2*, *MNSOD*, and *GLUT1* in buffalo pre-implantation embryos derived from IVF, and NT (AF and AFSC). **a** *OCT4*, **b** *NANOG*, **c** *STAT3*, **d** *DNMT*, **e** *DNMT3A*, **f** *HDAC2*, **g** *IGF2*, **h** *IGF2R*, **i** *BAX*, **j** *BCL2*, **k** *MNSOD*, **l** *GLUT1* mRNA from pools (triplicates) of 10 embryos each at the 4-cell, 8- to 16-cell; 5–6 embryos at the morulae, and blastocysts stages were reverse

(Fig. 2g). The RA of *IGF2R* was significantly ($P < 0.05$) higher at the 8- to 16-cell stage in the AF-NT embryos, whereas the levels were non significantly ($P > 0.05$) different between IVF and AFSC-NT embryos. No significant ($P > 0.05$) difference in the RA between IVF and NT embryos was observed at the blastocyst stage (Fig. 2h).

Expression of proapoptotic and antiapoptotic genes

Significantly ($P < 0.05$) higher RA of *BAX* was observed in AF-NT embryos followed by AFSC-NT and IVF embryos at all stages (Fig. 2i). Transcript abundance of *BCL2* was significantly ($P < 0.05$)

transcribed, and subjected to real-time quantitative PCR using transcript-specific primers. All reactions were conducted in triplicate, and normalized for *GAPDH* mRNA expression. Values are presented as mean $\pm$ SEM. Values with superscripts “abcd” refers to significant ($P < 0.05$) differences in relative transcript abundance between IVF, and NT embryos at a given stage of development

higher in IVF embryos at 8- to 16-cell as compared to NT embryos, whereas no significant ($P > 0.05$) differences were found between AF and AFSC-NT embryos. No significant ($P > 0.05$) differences were observed in the RA of *BCL* between AFSC-NT and IVF embryos at the blastocyst stage, whereas lower levels were detected in AF-NT embryos (Fig. 2j).

Oxidative stress related gene

At the 4-cell stage, *MnSOD* transcript level did not vary between IVF and NT embryos. At the 8- to 16-cell and morula stages, it was significantly ($P < 0.05$) higher in IVF embryos than NT

counterparts, whereas no significant ($P > 0.05$) difference was observed between AF and AFSC-NT embryos. No significant ($P > 0.05$) differences in RA of *MnSOD* transcript were observed between NT embryos at the blastocysts stage. Differences between IVF and AF-NT embryos were significant at the blastocyst stage, where the levels were significantly ($P < 0.05$) higher in AF-NT embryos (Fig. 2k).

Expression of glucose transport related gene

Transcription levels of *GLUT1* mRNA in IVF embryos showed higher levels at all stages than of NT counterparts, except at the 8- to 16-cell stage. No significant ($P > 0.05$) differences in RA of *GLUT1* transcript were observed between IVF and NT embryos at the 4-cell stage. At the 8- to 16-cell stage, the RA of *GLUT1* transcript were observed non significant ($P > 0.05$) between IVF and AFSC-NT embryos, whereas significantly ($P < 0.05$) higher levels were detected in AF-NT embryos. Less variation was observed between AF and AFSC-NT embryos at all stages except at the 8- to 16-cell stage (Fig. 2l).

Discussion

It has been shown that the use of several assisted reproductive technologies such as IVF and SCNT can affect embryo quality in terms of gene expression and epigenetic marks in oocytes and embryos as compared with in vivo-produced embryos (Niemann et al. 2002; Wrenzycki et al. 2007). We analyzed in the present studies a group of key genes involved in pre-implantation development in buffalo NT embryos reconstructed with AFs and AFSCs using in vitro fertilized embryos as control. It is likely that gene expression analysis at the pre-implantation stages for two different types of embryos with different developmental potential might offer better insight into the reprogramming process. It is possible that gene expression analysis at different stages of pre-implantation development in the present study might correspond to gene expression analysis before and after MZT between NT and IVF embryos. To the best of our knowledge, this is perhaps, the first report comparing mRNA expression patterns of development-related genes among pre-implantation buffalo NT embryos with different developmental

potentials. The genes under study were selected because they participate in key cellular processes during pre-implantation development and have been reported to become transcriptionally active during pre-implantation stage (Li et al. 2006a, b; Amarnath et al. 2007; Wrenzycki et al. 2007). The genes evaluated include genes involved in pluripotency and transcription (*OCT4*, *NANOG* and *STAT3*), chromatin structure (*HDAC2*), DNA methylation (*DNMT1* and *DNMT3A*), imprinting (*IGF2* and *IGF2R*), pro-apoptosis (*BAX*); anti-apoptosis (*BCL2*), oxidative stress (*MnSOD*), and metabolism (*GLUT1*).

The present study demonstrated that *OCT4* expression in NT embryos at the blastocyst stage was significantly decreased as compared to IVF embryos. It has been reported that maintenance of the pluripotent state depends on keeping *OCT4* expression between upper and lower limits; loss of expression leads to trophectoderm differentiation while a higher level induces differentiation to mesoderm and endoderm (Nichols et al. 1998; Niwa et al. 2000). It has also been opined that a low *OCT4* expression level at the blastocyst stage is associated with the low developmental potential of NT embryos (Li et al. 2005). The down-regulation of *OCT4* gene is considered to be a candidate marker for the low potential of NT embryos to develop healthy offspring (Kato and Tsunoda 2010). Our data indicate that an abnormal pattern of *OCT4* expression in NT embryos might lead to their compromised developmental competence. In the current study, different transcription patterns of *NANOG* were observed between embryos with a higher abundance in IVF derived embryos. A low level of *NANOG* expression induces insecurity in the pluripotency of ES cells, representing that *NANOG* functions to maintain pluripotency in a dose-dependent manner (Hatano et al. 2005). In addition, the expression of *NANOG* specifically corresponds to the pluripotent phenotype and, thus, is apparently present in the initial formation of the mouse blastocyst (Chambers et al. 2003). However, our results may be an indication that the molecular mechanisms activating the initial transcription of *NANOG* during development are different from those regulating maintenance of its expression in the pluripotent cell. We reasoned that a molecular understanding of the regulation of this gene in buffalo pre-implantation embryos would provide further insights into the maintenance of pluripotency. In the current study, apart from the blastocyst stage, a higher

abundance of *STAT3* was observed in NT embryos derived from AFs than from AFSCs. This is consistent with the observed up-regulation of *STAT3* mRNA expression at the 8-cell stage in fibroblast-NT embryos compared with both fetal skin cells and in vivo produced embryos in porcine (Zhu et al. 2004). Up-regulation of *STAT3* may influence cell proliferation (Zhu et al. 2004) and, interestingly, results of the present study showed an increase in the number of cells at the blastocyst stage in IVF and also to some extent in AFSC-NT embryos in contrast to its down-regulation and low cell number in AF-NT blastocysts.

The data of the present study revealed that, compared to IVF embryos, the RA of *DNMT1* transcript was significantly ($P < 0.05$) increased in NT embryos. These results indicated that buffalo NT embryos seemed to have unusually higher *DNMTs* mRNA transcripts than IVF embryos do, signifying that there existed relatively high levels of *DNMTs* activities during development in vitro for SCNT embryos (Suteevun et al. 2006). These abnormal levels of embryonic methylation may be associated with reduced developmental potential. It is likely that the results from different approaches used to evaluate methylation changes may provide varying information (Fulka et al. 2006). It was found that the *DNMT1* expression in NT group appeared significantly ($P < 0.05$) higher than that of IVF group at 4-cell and 8- to 16-cell stage, coincident with MZT. The *DNMT1* is responsible for cytosine methylation in mammals and influences gene silencing, the over expression of *DNMT1* is associated with repression of gene expression. For that reason, the higher expression of *DNMT1* during the MZT period in the present study may be leading to an incomplete activation of the embryonic genome. In addition, *DNMT3A* mRNA was expressed at all stages analyzed with a significant increase in abundance between the 8- to 16-cell and morula to blastocyst stages. Expression of *DNMT3A* in AFSC-NT and IVF blastocysts was similar, whereas it was lower in AF-NT blastocyst. This suggests that differential *DNMT3A* mRNA levels in AF-NT embryos have serious implications for the pre-implantation development and resulted in the lower number of blastocyst formation of AF derived embryos. Given the possible role in *de novo* methylation, altered level of *DNMT3A* expression might contribute to the imprinting problem observed in cloned embryos. Consistent with the above

observations of aberrant DNA methylation in embryos, the data of the present study suggested that the reprogramming events that occur during the development of the IVF produced embryos are likely to occur in NT produced embryos. Whether the consistently low efficiency of NT is related to the inability of a somatic nucleus to undergo the normal changes in methylation as indicated by increased levels of *DNMT1* or to the lack of *de novo* methylation triggered by low *DNMT3A* expression remains unclear in buffalo. However, further studies need to be carried out on the global levels of DNA methylation and histone acetylation in AF and AFSC-derived NT embryos, using IVF embryos as controls, before meaningful conclusions can be drawn in this species.

Results of the present study demonstrated that a high level of *HDAC2* mRNA at the 4-cell stage with a steady decrease, reaching a minimum at the morula and blastocyst stages in NT embryos. At the blastocyst stage *HDAC2* expression was similar between AFSC-NT embryos and IVF control embryos, whereas it was significantly lower in AF-NT embryos when compared to IVF control. This might be an indication of failed reprogramming of inefficient nuclei after transfer in the oocyte cytoplasm. According to Beyhan et al. (2007), donor cell efficiency has great impact on the *HDACs* mRNA expression in SCNT embryos, and this might be a reason for the differential expression in cloned blastocyst produced from different cell lines. Taken together, results of the present study indicate that the donor genome was unlikely reprogrammed to switch over the pattern of gene expression at the appropriate time from the somatic to the embryonic type following NT.

In this study, the expression of *IGF2* and *IGF2R* transcripts was significantly different in NT embryos compared to IVF counterparts. Alteration in mRNA expression of *IGF-2* between NT- and IVF-derived blastocysts can be associated with the inappropriate genomic imprinting because of difference in embryo production procedure (Wrenzycki et al. 2001; Dindot et al. 2004). It appears to be biallelically transcribed up to the morula stage, but in the blastocyst stage the maternal *IGF2* allele is silenced (Mizuki et al. 2001). Thus, it appears that the NT embryos suffer a loss of imprinting and, therefore, overexpress *IGF2*. In addition, in vitro manipulation might induce epigenetic alterations in various developmentally important genes (Niemann et al. 2002) and studies have shown

that such epigenetic mutations particularly affect the expression of genes that are regulated by genomic imprinting (Feil 2001). Consistent with the hypothesis of Haig and Graham (1991), which predicts that imprinting of growth factors such as *IGF2* and *IGF2R* regulates embryonic growth in the mammals. However, information with regard to imprinting of these genes in buffalo is scarce.

The data from the current study indicate that, *BAX* expression showed a steady increase through the developmental stages and the levels were significantly ($P < 0.05$) higher in NT embryos from AFs than in embryos from AFSCs and IVF, suggesting that overexpression of the *BAX* gene accelerates cellular apoptotic pathways (Yang and Rajamahendran 2002; Opiela et al. 2008). This non-regulation of *BAX* mRNA production could be a cause for the low quality of the cloned embryos. Another finding from this experiment was that expression level of *BCL2* mRNA of AF-NT embryos was significantly ($P < 0.05$) lower than those of IVF and AFSC embryos at the blastocyst stage. *BCL2* is considered to be an anti-apoptotic gene, and its expression level should be low when apoptotic incidence is high. Transcript abundance of cell death regulatory genes may be altered in embryos undergoing fragmentation, so that expression of the gene involved in cell death (*BAX*) is elevated, and expression of the gene involved in cell survival (*BCL2*) may be reduced. Yang and Rajamahendran (2002) demonstrated that good quality embryos have a greater concentration of *BCL2* protein than that of *BAX* protein, while there is more *BAX* than *BCL2* in lower quality embryos. The higher apoptotic incidence of SCNT embryos than those of the IVF group may be attributed to the in vitro micromanipulation (McElroy et al. 2008). The in vitro micromanipulation could cause cellular mechanical damages to some extent, resulting in higher apoptotic rate of buffalo SCNT reconstructed embryos compared with IVF embryos. This could be another demonstration that in vitro manipulation may reduce embryo development, and increase the incidence of apoptosis (McElroy et al. 2008). On the other hand, the higher *BCL2* mRNA expression of SCNT embryos at the blastocyst stage may be the active response of SCNT embryos to deal with the manipulation stress in vitro, suggesting that the embryos have a higher capability of response to the mechanical stimuli of in vitro manipulation at later developmental stages. However, the capability of response decreased during the early embryonic

development. In this study, the expression pattern of *BCL2* in NT embryos reflected the results observed in our previous studies with respect to total cell number and developmental potential. Our findings support the concept proposed by Oltvai et al. (1993) that the ratio of *BCL2* to *BAX* determines whether a cell lives or dies. However, it is also known that gene expression can be dramatically affected by the in vitro culture environment and can undergo considerable alteration without arresting development (Wrenzycki et al. 2001; Arias et al. 2012). Although the importance of mRNA expression profiles of apoptosis-related genes in the buffalo NT embryos, little information is available on this aspect.

In the current study, the higher expression of *MnSOD* in the IVF-derived 8- to 16-cell, morula and blastocyst stages embryos compared with either type of NT embryos might be due to early activation of this gene during IVF at the minor MZT (Memili et al. 1998), which occurs at the 2–4 cell stage in bovine. In contrast to the expression pattern at the 8- to 16-cell and morula stages, *MnSOD* expression was higher in the NT blastocysts, particularly in the AF-NT blastocysts compared with IVF counterparts. Although not significant, the expression level was relatively higher in the AFS-NT blastocysts compared with the IVF counterparts. Because *MnSOD* is implicated in promoting cellular differentiation (St.Clair et al. 1994), the higher expression in NT blastocyst could be due to inadequate reprogramming or de-differentiation of the donor genome in the NT embryos. The aberrant expression of this gene at the 8- to 16-cell, morula and blastocyst stages in NT embryos suggests that NT embryos fail to establish an appropriate embryonic *MnSOD* expression pattern. These results are contradictory to Kim et al. (2012), who described no differences in the expression levels of *MnSOD* between bovine SCNT and IVF embryos. This divergence among experiments may be due to different protocols used to produce and culture embryos, different methods to assess gene expression profile.

There were significant differences in the expression of *GLUT1* between the NT and IVF embryos. Studies in mouse model suggest that with in vivo embryos, *GLUT1* decreased 50 % in in vitro blastocysts (Morita et al. 1992). These reports clearly suggest that viable embryos have higher *GLUT1* expression. The decrease in the relative expression of *GLUT1* in AF-NT blastocyst supports the notion that AF-NT embryos are inferior to

IVF embryos in their ability to develop them. Decreased glucose transporter expression triggers *BAX*-dependent apoptosis in murine blastocysts (Chi et al. 2000); hence it is possible that, the low level of *GLUT1* expression observed in the present study in AF-NT embryos is usually related to higher levels of apoptotic incidence in these embryos at blastocyst stage.

The current study demonstrates the differential expression pattern of genes concerned in transcription, pluripotency, chromatin structure, DNA methylation, growth factor signalling and imprinting, apoptosis, oxidative stress and metabolism during pre-implantation development of NT embryos compared to their IVF derived counterparts. Additionally, the variations in the RA of target transcripts between IVF and NT embryos may support the fact in buffalo, that the method of embryo generation has considerable influence on the transcriptional levels of the resulting embryos at different stages of development as reported earlier in bovine (Farin et al. 2004; Wrenzycki et al. 2004; Rodríguez-Alvarez et al. 2010). In this study, the RA of target transcripts in AFSC-NT embryos more directly followed that of the IVF derived embryos compared with AF-NT embryos. The different expression levels of these genes have serious implications for the subsequent development of NT-derived embryos produced from these two types of cells because this was further supported by our previous study results of an enhanced pre-implantation development with increased cell number in AFSC-NT embryos as compared to AF-NT counterparts. The low developmental competence of pre-implantation AF-NT embryos may, at least in part, be due to inadequate reprogramming leading to defects in early embryonic gene expression. It is also likely that the abnormal expression of target transcripts had a collective effect and was accountable, at least to some extent, for the lower developmental potential of AF-NT embryos compared with AFSC-NT and IVF embryos. In conclusion, these findings, therefore, suggest that AFSCs may be more efficiently reprogrammed to reactivate expression of early embryonic genes than AFs and thereby AFSCs with relatively undifferentiated genome might serve as efficient donors for NT.

Acknowledgments The authors would like to thank Dr. BS Punia, ICAR-Central Institute for Research on Buffaloes, for reviewing the manuscript. Funding support from Indian Council of Agricultural Research (ICAR), New Delhi is gratefully acknowledged.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

- Amarnath D, Li X, Kato Y, Tsunoda Y (2007) Gene expression in individual bovine somatic cell cloned embryos at the 8-cell and blastocyst stages of pre-implantation development. *J Reprod Dev* 53:1247–1263
- Arias ME, Sanchez R, Felmer R (2012) Evaluation of different culture systems with low oxygen tension on the development, quality and oxidative stress-related genes of bovine embryos produced in vitro. *Zygote* 20:209–217
- Arnold DR, Bordignon V, Lefebvre R, Murphy BD, Smith LC (2006) Somatic cell nuclear transfer alters peri-implantation trophoblast differentiation in bovine embryos. *Reproduction* 132:279–290
- Beyhan Z, Forsberg EJ, Eilertsen KJ, Kent-First M, First NL (2007) Gene expression in bovine nuclear transfer embryos in relation to donor cell efficiency in producing live offspring. *Mol Reprod Dev* 74:18–27
- Brackett BG, Oliphant G (1975) Capacitation of rabbit spermatozoa in vitro. *Biol Reprod* 12:260–274
- Castro FO, Sharbati S, Rodríguez-Alvarez LL, Cox JF, Hultschig C, Einspanier R (2010) MicroRNA expression profiling of elongated cloned and in vitro-fertilized bovine embryos. *Theriogenology* 73:71–85
- Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A (2003) Functional expression cloning of *NANOG*, a pluripotency sustaining factor in embryonic stem cells. *Cell* 113:643–655
- Cheong HT, Takahashi Y, Kanagawa H (1993) Birth of mice after transplantation of early cell-cycle-stage embryonic nuclei into enucleated oocytes. *Biol Reprod* 48:958–963
- Chi MM, Pingsterhaus J, Carayannopoulos M, Moley KH (2000) Decreased glucose transporter expression triggers *BAX*-dependent apoptosis in the murine blastocyst. *J Biol Chem* 275:40252–40257
- Daniels R, Hall V, Trounson AO (2000) Analysis of gene transcription in bovine nuclear transfer embryos reconstructed with granulosa cell nuclei. *Biol Reprod* 63:1034–1040
- de A Camargo LS, Powell AM, do Vale Filho VR, Wall RJ (2005) Comparison of gene expression in individual pre-implantation bovine embryos produced by in vitro fertilization or somatic cell nuclear transfer. *Reprod Fertil Dev* 17:487–496
- Dindot SV, Farin PW, Farin CE, Romano J, Walker S, Long C, Piedrahita JA (2004) Epigenetic and genomic imprinting analysis in nuclear transfer derived *Bos gaurus*/*Bos taurus* hybrid fetuses. *Biol Reprod* 71:470–478
- Farin CE, Farin PW, Piedrahita JA (2004) Development of fetuses from in vitro-produced and cloned bovine embryos. *J Anim Sci* 82:53–62
- Feil R (2001) Early embryonic culture and manipulation could affect genomic imprinting. *Trends Mol Med* 7:245–246
- Fulka J, Fulka H, Slavik T, Okada K, Fulka J Jr (2006) DNA methylation pattern in pig in vivo produced embryos. *Histochem Cell Biol* 126:213–217

- Garry FB, Adams R, McCann JP, Odde KG (1996) Postnatal characteristics of calves produced by nuclear transfer cloning. *Theriogenology* 45:141–152
- George A, Sharma R, Singh KP, Panda SK, Singla SK, Palta P, Manik R, Chauhan MS (2011) Production of cloned and transgenic embryos using buffalo (*Bubalus bubalis*) embryonic stem cell-like cells isolated from in vitro fertilized and cloned blastocysts. *Cell Reprogram* 13:263–272
- Giraldo AM, Lynn JW, Purpera MN, Godke RA, Bondioli KR (2007) DNA methylation and histone acetylation patterns in cultured bovine fibroblasts for nuclear transfer. *Mol Reprod Dev* 74:1514–1524
- Haig D, Graham C (1991) Genomic imprinting and the strange case of the insulin-like growth factor receptor. *Cell* 64:1045–1046
- Hatano SY, Tada M, Kimura H, Yamaguchi S, Kono T, Nakano T, Suemori H, Nakatsuji N, Tada T (2005) Pluripotential competence of cells associated with *NANOG* activity. *Mech Dev* 122:67–79
- Hiiragi T, Solter D (2005) Reprogramming is essential in nuclear transfer. *Mol Reprod Dev* 70:417–421
- Jousan FD, De Castro LA, Brad AM, Roth Z, Hansen PJ (2008) Relationship between group II caspase activity of bovine pre-implantation embryos and capacity for hatching. *J Reprod Dev* 54:217–220
- Kanka J (2003) Gene expression and chromatin structure in the pre-implantation embryo. *Theriogenology* 59:3–19
- Kato Y, Tsunoda Y (2010) Role of donor nuclei in cloning efficiency: can the ooplasm reprogram any nucleus. *Int J Dev Biol* 54:1623–1629
- Kato Y, Li XP, Amarnath D, Uuhizawa K, Hashizume K, Tokunaga T, Taniguchi M, Tsunoda Y (2007) Comparative gene expression analysis of bovine nucleartransferred embryos with different developmental potential by cDNA microarray and real-time PCR to determine genes that might reflect calf normality. *Cloning Stem Cells* 9:495–511
- Kim HS, Lee JY, Jeong EJ, Yang CJ, Hyun SH, Shin T, Hwang WS (2012) Effects of repetitive ionomycin treatment on in vitro development of bovine somatic cell nuclear transfer embryos. *J Reprod Dev* 58:132–139
- Li X, Kato Y, Tsunoda Y (2005) Comparative analysis of development related gene expression in mouse pre-implantation embryos with different developmental potential. *Mol Reprod Dev* 72:152–160
- Li XP, Amarnath D, Kato Y, Tsunoda Y (2006a) Analysis of development related gene expression in cloned bovine blastocysts with different developmental potential. *Cloning Stem Cells* 8:41–50
- Li XP, Kato Y, Tsunoda Y (2006b) Comparative studies on the mRNA expression of development-related genes in an individual mouse blastocyst with different developmental potential. *Cloning Stem Cells* 8:214–224
- Li X, Kato Y, Tsuji Y, Tsunoda Y (2008) The effects of trichostatin A on mRNA expression of chromatin structure-, DNA methylation-, and development-related genes in cloned mouse blastocysts. *Cloning Stem Cells* 10:133–142
- Loneragan P, Rizos D, Gutiérrez-Adán A, Moreira PM, Pintado B, de la Fuente J, Boland MP (2003a) Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage in vitro or in vivo. *Biol Reprod* 69:1424–1431
- Loneragan P, Rizos D, Gutierrez-Adan A, Fair T, Boland MP (2003b) Oocyte and embryo quality: effect of origin, culture conditions and gene expression patterns. *Reprod Domest Anim* 38:259–267
- McElroy SL, Kim JH, Kim S, Jeong YW, Lee EG, Park SM, Hossein MS, Koo OJ, Abul Hashem MD, Jang G, Kang SK, Lee BC, Hwang WS (2008) Effects of culture conditions and nuclear transfer protocols on blastocyst formation and mRNA expression in pre-implantation porcine embryos. *Theriogenology* 69:416–425
- Memili E, Dominko T, First NL (1998) Onset of transcription in bovine oocytes and pre-implantation embryos. *Mol Reprod Dev* 51:36–41
- Mizuki O, Nao A, Hiroyuki S (2001) Allele specific detection of nascent transcripts by fluorescence in situ hybridization reveals temporal and culture-induced changes in *IGF2* imprinting during pre-implantation mouse development. *Genes Cells* 6:249–259
- Mizutani E, Ono T, Li C, Maki-Suetsugu R, Wakayama T (2008) Propagation of senescent mice using nuclear transfer embryonic stem cell lines. *Genesis* 46:478–483
- Morita Y, Tsutsumi O, Hosoya I, Taketani Y, Oka Y, Kato T (1992) Expression and possible function of glucose transporter protein *GLUT1* during pre-implantation mouse development from oocytes to blastocysts. *Biochem Biophys Res Commun* 188:8–15
- Munoz M, Rodriguez A, De Frutos C, Caamano JN, Diez C, Facal N, Gomez E (2008) Conventional pluripotency markers are unspecific for bovine embryonic-derived cell-lines. *Theriogenology* 69:1159–1164
- Nichols J, Zevnik B, Anastasiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Schöler H, Smith A (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the *POU* transcription factor *OCT4*. *Cell* 95:379–391
- Niemann H, Wrenzycki C (2000) Alterations of expression of developmentally important genes in pre-implantation bovine embryos by in vitro culture conditions: implications for subsequent development. *Theriogenology* 53:21–34
- Niemann H, Wrenzycki C, Lucas-Hahn A, Brambrink T, Kues WA, Carnwath JW (2002) Gene expression patterns in bovine in vitro-produced and nuclear transfer-derived embryos and their implications for early development. *Cloning Stem Cells* 4:29–38
- Niwa H, Miyazaki J, Smith AG (2000) Quantitative expression of *OCT3/4* defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* 24:372–376
- Oltvai ZN, Millman CL, Korsmeyer SJ (1993) *BCL2* heterodimerizes in vitro with a conserved homology, *BAX*, that accelerates programmed cell death. *Cell* 74:609–619
- Opiela J, Katska-Ksiazkiewicz L, Lipinski D, Slomski R, Bzowska M, Rynska B (2008) Interactions among activity of glucose-6-phosphate dehydrogenase in immature oocytes, expression of apoptosis-related genes *BCL2* and *BAX*, and developmental competence following IVP in cattle. *Theriogenology* 69:546–555
- Ranilla MJ, Gebbie FE, King ME, Carolan C, Sinclair KD, Watt RG, Dolman DF, Beckers JF, Robinson JJ (1998) The incidence of embryo and fetal loss following the transfer of in vitro-cultured sheep embryos. *Theriogenology* 49:248
- Rodríguez-Alvarez L, Cox J, Tovar H, Einspanier R, Castro FO (2010) Changes in the expression of pluripotency-

- associated genes during pre-implantation and peri-implantation stages in bovine cloned and in vitro produced embryos. *Zygote* 18:269–279
- Roth Z, Hansen PJ (2002) Disruption of nuclear maturation and rearrangement of cytoskeletal elements in bovine oocytes exposed to heat shock during maturation. *Reproduction* 129:235–244
- Sadeesh EM, Meena K, Balhara S, Yadav PS (2014a) Expression profile of developmental important genes between hand-made cloned buffalo embryos produced from reprogramming of donor cell with oocytes extract and selection of recipient cytoplasm through brilliant cresyl blue staining and in vitro fertilized embryos. *J Assist Reprod Genet* 31:1541–1552
- Sadeesh EM, Meena K, Fozia S, Yadav PS (2014b) A comparative study on efficiency of adult fibroblasts and amniotic fluid-derived stem cells for production of hand-made cloned buffalo (*Bubalus bubalis*) embryos. *Cytotechnology*. doi:10.1007/s10616-014-9805-1
- Shah R, George A, Singh MK, Kumar D, Chauhan MS, Manik RS, Palta P, Singla SK (2008) Hand-made cloned buffalo (*Bubalus bubalis*) embryos: comparison of different media and culture systems. *Cloning Stem Cells* 10:435–442
- Shah RA, George A, Singh MK, Kumar D, Anand T, Chauhan MS, Manik RS, Palta AP, Singla SK (2009) Pregnancies established from hand-made cloned blastocyst reconstructed using skin fibroblast in buffalo (*Bubalus bubalis*). *Theriogenology* 71:1215–1219
- Shi D, Lu F, Wei Y, Cui Y, Yang S, Wei J, Liu Q (2007) Buffalos (*Bubalus bubalis*) cloned by nuclear transfer of somatic cells. *Biol Reprod* 77:285–291
- Soliman MK (1975) Studies on physiological chemistry of allantoic fluid of buffalo at various periods of pregnancy. *Indian Vet J* 52:106–112
- Srirattana K, Lorthongpanich C, Laowtammathro C, Imsoonthornruksa S, Ketudat-Cairns M, Phermthai T, Nagai T, Parnpai R (2010) Effect of donor cell types on developmental potential of cattle (*Bos taurus*) and swamp buffalo (*Bubalus bubalis*) cloned embryos. *J Reprod Dev* 56:49–54
- St.Clair DK, Oberley TD, Muse KE, St Clair WH (1994) Expression of manganese superoxide dismutase promotes cellular differentiation. *Free Radic Biol Med* 16:275–282
- Steuerwald N, Cohen J, Herrera RJ (1999) Analysis of gene expression in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT-PCR. *Mol Hum Reprod* 5:1034–1039
- Suteevun T, Smith SL, Muenthaisong S, Yang X, Parnpai R, Tian XC (2006) Anomalous mRNA levels of chromatin remodeling genes in swamp buffalo (*Bubalus bubalis*) cloned embryos. *Theriogenology* 65:1704–1715
- Vajta G, Lewis IM, Trounson AO, Purup S, Maddox-Hyttel P, Schmidt M, Pedersen HG, Greve T, Callesen H (2003) Hand-made somatic cell cloning in cattle: analysis of factors contributing to high efficiency in vitro. *Biol Reprod* 68:571–578
- Wakayama T, Rodriguez I, Perry AC, Yanagimachi R, Mombaerts P (1999) Mice cloned from embryonic stem cells. *Proc Natl Acad Sci USA* 96:14984–14989
- Wakayama S, Mizutani E, Kishigami S, Thuan NV, Ohta H, Hikichi T, Bui HT, Miyake M, Wakayama T (2005) Mice cloned by nuclear transfer from somatic and nES cells derived from the same individuals. *J Reprod Dev* 51:765–772
- Warzych E, Wrenzycki C, Peippo J, Lechniak D (2007) Maturation medium supplements affect transcript level of apoptosis and cell survival related genes in bovine blastocysts produced in vitro. *Mol Reprod Dev* 74:280–289
- Wrenzycki C, Nieman H (2003) Epigenetic reprogramming in early embryonic development: effects of in vitro production and somatic nuclear transfer. *Reprod Biomed Online* 7:649–656
- Wrenzycki C, Wells D, Herrmann D, Miller A, Oliver J, Tervit R (2001) Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. *Biol Reprod* 65:309–317
- Wrenzycki C, Herrmann D, Lucas-Hahn A, Lemme E, Korsawe K, Niemann H (2004) Gene expression patterns in in vitro-produced and somatic nuclear transfer-derived pre-implantation bovine embryos: relationship to the large offspring syndrome? *Anim Reprod Sci* 82–83:593–603
- Wrenzycki C, Herrmann D, Carnwath JW, Niemann H (2007) Messenger RNA in oocytes and embryos in relation to embryo viability. *Theriogenology* 68:77–83
- Yang MY, Rajamahendran R (2002) Expression of *BCL-2* and *BAX* proteins in relation to quality of bovine oocytes and embryos produced in vitro. *Anim Reprod Sci* 70:159–169
- Zhang S, Kubota C, Yang L, Zhang Y, Page R, O'Neill M, Yang X, Tian XC (2004) Genomic imprinting of H19 in naturally reproduced and cloned cattle. *Biol Reprod* 71:1540–1544
- Zhao XE, Zheng YM (2010) Development of cloned embryos from porcine neural stem cells and amniotic fluid-derived stem cells. *Animal* 4:921–992
- Zhu H, Craig JA, Dyce PW, Sunnen N, Li J (2004) Embryos derived from porcine skin-derived stem cells exhibit enhanced pre-implantation development. *Biol Reprod* 71:1890–1897