Differential developmental competence and gene expression patterns in buffalo (*Bubalus bubalis*) nuclear transfer embryos reconstructed with fetal fibroblasts and amnion mesenchymal stem cells

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Abstract The developmental ability and gene expression pattern at 8- to 16-cell and blastocyst stages of buffalo (*Bubalus bubalis*) nuclear transfer (NT) embryos from fetal fibroblasts (FFs), amnion mesenchymal stem cells (AMSCs) and in vitro fertilized (IVF) embryos were compared in the present studies. The in vitro expanded buffalo FFs showed a typical “S” shape growth curve with a doubling time of 41.4 h and stained positive for vimentin. The in vitro cultured undifferentiated AMSCs showed a doubling time of 39.5 h and stained positive for alkaline phosphatase, and these cells also showed expression of pluripotency markers (*OCT4*, *SOX2*, *NANOG*), and mesenchymal stem cell markers (*CD29*, *CD44*) and were negative for haematopoietic marker (*CD34*) genes at different passages. Further, when AMSCs were exposed to corresponding induction conditions, these cells differentiated into adipogenic, chondrogenic and osteogenic lineages which were

confirmed through oil red O, alcian blue and alizarin staining, respectively. Donor cells at 3–4 passage were employed for NT. The cleavage rate was significantly ($P < 0.05$) higher in IVF than in FF-NT and AMSC-NT embryos (82.6 ± 8.2 vs. 64.6 ± 1.3 and 72.3 ± 2.2 %, respectively). However, blastocyst rates in IVF and AMSC-NT embryos (30.6 ± 2.7 and 28.9 ± 3.1 %) did not differ and were significantly ($P < 0.05$) higher than FF-NT (19.5 ± 1.8 %). Total cell number did not show significant ($P > 0.05$) differences between IVF and AMSC-NT embryos (186.7 ± 4.2 , 171.2 ± 3.8 , respectively) but were significantly ($P < 0.05$) higher than that from FF-NT (151.3 ± 4.1). 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*), metabolism (*GLUT1*) and oxidative stress (*MnSOD*) regulation were observed in cloned embryos. The transcripts or expression patterns in AMSC-NT embryos more closely followed that of the in vitro derived embryos compared with FF-NT embryos. The results demonstrate that multipotent amnion MSCs have a greater potential as donor cells than FFs in achieving enhanced production of cloned buffalo embryos.

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Introduction

State of donor cells is one of the most significant 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 nuclear transfer–ES (NT–ES) cells were used as donor cells 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 can be successfully used for NT in mice, this process is limited in other species because 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 on 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; Hiragi 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). These results show that more research is

required to search a better donor cell type which can support cloned embryo production with better pregnancy rate and development to the term of cloned buffalo embryos in spite of the poor viability of buffalo NT embryos, with an exceptionally low rate of cloned buffalo calf production.

Evidence indicates that the use of bone marrow mesenchymal stem cells (BM-MSCs) as nuclear donors in somatic cell nuclear transfer (SCNT) increases the embryonic developmental rates of cloned domestic animals such as pigs and bovine (Colleoni et al. 2005; Lee et al. 2010). Although BM-MSCs represent the most widely investigated mesenchymal stem cells for application in nuclear transfer to date, these cells have more limited potential than embryonic stem cells in terms of both in vitro proliferation ability (about 32 days for expansion between isolation and implantation) and target differentiation capacity (Guest et al. 2010), and thus do not appear to improve long-term functionality noticeably (Paris and Stout 2010). Meanwhile, human BM-MSCs show reduced plasticity and growth with increasing donor age and in vitro passage number (Guillot et al. 2007). Therefore, progenitor cells derived from extra-fetal sources may be alternative candidates with the potential to circumvent many of these limitations, and thus opening new perspectives for developmental biology and regenerative medicine.

It is generally accepted that mammalian amnion is derived from the extra-embryonic endoderm or extra-embryonic trophoderm. The amniotic membrane (AM) is composed of three major layers: a single epithelial layer, an avascular mesenchyme and a thick basement membrane. The compact layer of stromal matrix adjacent to the basement membrane forms the fibrous skeleton of the AM. It is adjacent to the trophoblast cells and lines the amniotic cavity. Amnion can be separated from the underlying chorion, as chorion and amnion are not truly fused at cellular level, because AM has anti-adhesive properties and is felt to promote epithelialisation and decrease inflammation, neovascularization, and fibrosis. The AM has been regarded as a promising source of non-invasive isolation of MSCs, and poses no ethical concerns as fetal membranes are discarded at birth. Advantages of amnion over other sources for stem cells included abundant availability, ethically non-objectionable and non-invasive source. The MSCs from AM (AMSCs) are thought to be in an intermediate stage between

embryonic stem cells and lineage-restricted adult stem cells (In't Anker et al. 2004; De Coppi et al. 2007; Gucciardo et al. 2009). Recently, Yamahara et al. (2014) reported that mesenchymal stem cells isolated from human amniotic tissue have an angiogenesis potential. In animals, amnion-derived stem cells have potential of differentiation into multilineage mesenchymal cell types in many species such as bovine (Corradetti et al. 2013), canine (Park et al. 2012), feline (Rutigliano et al. 2013), chicken (Gao et al. 2012), rat (Marcus et al. 2008) and equine (Coli et al. 2011; Violini et al. 2012; Seo et al. 2013). Further, the amnion derived MSCs are reported to express certain key pluripotency markers including *OCT4* (Corradetti et al. 2013). The in vitro proliferative ability and expression of key pluripotency markers indicate the possibilities of using AMSCs in cloning technique for the agricultural field or for basic research. The gene expression profile demonstrated that certain genes in embryos cloned with MSCs closely resembled those of in vivo counter parts (Kumar et al. 2007). The MSC used in those studies were derived from tissues, such as bone marrow which cannot be sourced as easily as amnion tissue due to practical limitations. Term amnion of large animal is an easily available, plentiful, inexpensive and noninvasive source of multipotent MSCs with no ethical concern. Thus, the use of AMSC as nuclear donors is an increasingly interesting option because it may lead to an augmentation in the efficiency of animal cloning.

Therefore the present study was conducted to characterize an isolated population of MSCs from buffalo amnion by assessing their multilineage potential in order to compare the developmental potential and quality of embryos cloned with MSCs and fetal fibroblasts (FFs). To gain more insights into the relation between the state of differentiation and cloning efficiency, the gene expression patterns were also assessed, and compared with those of pre-implantation in vitro produced embryos as controls.

Materials and methods

Ethics statement, chemicals and reagents

These studies were conducted in accordance with the guidelines laid down by the Committee for the Purpose of Control and Supervision on Experiments

on Animals (CPCSEA) and with the approval from the Institute Animal Ethics Committee (IAEC). All chemicals, reagents, culture media were of cell culture grade and obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. Fetal bovine serum (FBS) was from Hyclone (Thermo Scientific, Wilmington, DE, USA), with the same batch used 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 fetal fibroblast cells (FFs) culture

Buffalo gravid uteri at 50–100 days gestation were obtained from abattoir, washed 2–3 times with isotonic saline fortified with 400 IU/ml penicillin and 500 µg/ml streptomycin and transported to the laboratory within 6 h. The fetus was located by uterine incision and taken out. The tissue was collected from fetus (n = 6; male = 3, female = 3) and cells were cultured in three replicates for each fetus processed. The fetal sub-dermal biopsies were taken from upper part of foreleg and minced by surgical blade into smaller pieces and washed 4–6 times with Dulbecco's phosphate buffered saline (DPBS). The tissue pieces were transferred on re-calcified buffalo plasma droplets (20 µl) in 25-cm² cell culture flasks. After placing the tissue pieces on the drops, it was allowed to coagulate for 30 min at 37 °C for the attachment of tissue to the surface of the culture flask. The adhered tissue pieces were cultured in culture medium containing Dulbecco's modified Eagle's medium (DMEM) with 10 % FBS, 2 mM L-glutamine, 1 % (v/v) nonessential amino acids, 1 % (v/v) vitamins, 1 % penicillin/streptomycin/amphotericin (Gibco, Life Technologies, Grand Island, NY, USA) in a CO₂ incubator (5 % CO₂ in humidified 95 % air at 38 °C). Cell outgrowth from the tissues was observed on the 4th and 5th days and cells were harvested when their confluence reached approximately 80–90 %. Cryogenic preservation was performed based on the method described earlier by Sadeesh et al. (2014a). Briefly, cells at exponential growth phase, which had

reached approximately 70–80 % confluence, were selected 24 h before cryogenic preservation, and the medium was refreshed. Cells were harvested and viability was checked with trypan blue staining before freezing. A half portion of the cell pellet obtained by centrifugation ($200\times g$, 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, 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.

Immunocytochemical characterization of fetal fibroblast cells

Immunofluorescence analyses were performed in cultured fetal fibroblast cells based on the procedure described earlier by Sadeesh et al. (2014a).

Growth kinetics

For the estimation of growth kinetics, FFs were seeded into 24-well plates (1×10^4 cells per well). All experiments were performed in duplicate at each time point and cell growth data were recorded until a plateau phase was reached. Determinations were made according to the method described earlier by Liu et al. (2008). The growth curve was plotted with these data and the population doubling time (PDT) was calculated from the curve.

Isolation and culture of AMSCs

Gravid bubaline uteri of first trimester were collected from abattoir and brought to laboratory within 4–5 h of slaughtering. The uterus was washed using sterile saline and uterine wall was incised under aseptic conditions to expose fetal membranes. Amnion was separated from chorion by peeling. The tissue was disintegrated by partial trypsinization (0.05 % trypsin–EDTA solution, 45 min at 37 °C). The cell clumps were allowed to settle for 5 min at room temperature. The cells in suspension were transferred into another tube and cell pellet was obtained by centrifugation at $450 \times g$ for 10 min. The cells were washed twice with PBS and re-suspended in culture medium (DMEM containing 10 % FBS, 1 % non-essential amino acids, 1 % vitamins, 1 % penicillin/

streptomycin/ampicillin). Cells were seeded at a density of 1×10^6 cells/ml in 25 cm² cell culture flasks under humidified conditions at 38.5 ± 0.5 °C in 5 % CO₂. After 7 days of culture, the medium was replaced with fresh one, and was subsequently replaced 3 times a week. At 80 % confluence, the flasks were washed to remove dead cells and debris; the cells were harvested with 0.05 % trypsin–EDTA for 5 min at 38.5 °C and plated again at 1×10^6 /ml. At 70 % of confluence, cells were used for stemness characterization and for differentiation experiments. One half portions of the cells were cryopreserved from passages 3–4 in 10 % DMSO in DMEM supplemented with 30 % FBS using the same cryopreservation protocol as described for FFs, whereas rests of the cells were cultured to grow further from each passage. Passage 3–4 was the last time point included for characterization and differentiation studies.

Growth kinetics

For determining the growth rate of AMSCs, the same culture protocol was used as described for the FFs.

Characterization of AMSCs

Alkaline phosphatase (AP) expression

The cultured cells were subjected to alkaline phosphatase staining using an alkaline phosphatase (AP) staining kit (Sigma, Chemical Co., #86C) as per manufacturers' instructions. Briefly, the medium was removed, and monolayer of AMSCs was washed twice with PBS. The cells were fixed in citrate-acetone-formaldehyde fixative for 1 min, washed three times with de-ionized water and incubated at room temperature for 15 min in the presence of alkaline dye under dark condition. The cells were rinsed again using de-ionized water, counterstained with neutral red and observed under inverted microscope (Nikon Inc., Tokyo, Japan) for AP staining. Cells with red stain were considered AP positive.

Expression of pluripotency and stem cell surface markers

Expression of pluripotency marker genes (*OCT-4*, *NANOG* and *SOX-2*), mesenchymal stem cell surface marker genes (*CD29* and *CD44*) and haematopoietic marker (*CD34*) were analysed using reverse transcriptase PCR (RT-PCR). In brief, total RNA was extracted

from AMSCs using the Cells-to-cDNA kit (Ambion Inc, The RNA Company, Austin, TX, USA) according to the manufacturers' instructions. The cells were washed with 200 μ l ice cold PBS after which 50 μ l of chilled cell lysis buffer was added and the mixture was incubated at 70 °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 μ l of the cell lysates (RNA), 4 μ l dNTP mix (2.5 mM each dNTP) and 2 μ l random decamer were put in a PCR tube. The reaction mixture was mixed and incubated at 70 °C for 1 min to denature RNA for easier binding of primer in a thermal cycler. The tubes were cooled immediately on ice and remaining reverse transcriptase reagents i.e. 2 μ l 10X RT buffer, 1 μ l MMLV and 1 μ l 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.

PCR reaction was carried out in a 50 μ l final volume containing 45 μ l platinum PCR supermix (Invitrogen, Carlsbad, CA, USA) and 5 μ l of primer (200 nM each, Sigma, St. Louis, MO, USA) and template DNA solution. A set of reaction without template cDNA was used as negative control for PCR reaction. GAPDH was used as the reference gene. The primer sequences used for *GAPDH*, *OCT4*, *NANOG*, *SOX2*, *CD29*, *CD44* and *CD34* are mentioned in Table 1. The PCR conditions were the same except for the annealing temperature (Table 1), as 94 °C for 2 min (initial denaturation), denaturation at 94 °C for 30 s, elongation at 72 °C for 1 min (35 cycles). The amplified DNA fragments were resolved on 2 % agarose gel containing 0.5 μ g/ml ethidium bromide against a 50-bp ladder and visualized under gel documentation system (Alpha Imager, Alpha Innotech, San Leandro, CA, USA).

In vitro induced differentiation

For differentiation, cultured cells were detached by trypsinization, centrifuged and then cultured in lineage-specific differentiation media for 3 weeks. The cells were seeded in tissue culture grade six-well

plates. To induce adipogenic differentiation, the cells were cultured in DMEM supplemented with 10 % FBS, 1 μ M dexamethasone, 500 μ M isobutylmethylxanthine, 60 μ M indomethacin and 5 μ g/ml insulin. The presence of intracellular lipid globules indicative of adipogenic differentiation was assessed by staining cells with oil red O solution on day 21. The medium was replaced twice a week. Chondrogenic differentiation was induced in confluent monolayer cultures of AMSCs using a specific chondrogenesis differentiation kit (StemPro, Invitrogen). Differentiated cells were stained with alcian blue 8GX (Sigma-Aldrich) on day 21. For osteogenic differentiation, DMEM supplemented with 10 % FBS, 100 nM dexamethasone, 50 μ M ascorbic acid and 10 mM β -glycerol phosphate was used. The differentiation of cells was assessed morphologically and they were stained with alizarin red which indicates the calcium mineralization in cells. Non induced control cells were cultured for the same time in standard control medium (DMEM supplemented with 15 % FBS, 1 % non-essential amino acids, 1 % vitamins, 1 % pen/strep/amp).

Preparation of donor cells

Donor cells (FFs and AMSCs) at 3–4 passage were employed for NT. 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 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 $\times$ 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 μ g/ml gentamicin and the following 20 number denotes 20 % FBS) for use as nucleus donor cells.

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

Gene name		Sequence (5'–3')	Annealing temp (°C)	Gene Bank accession no.
<i>GAPDH</i>	Forward	CCTGCCAAGTATGATGAGA	53	GU324291
	Reverse	GAAGGTAGAAGAGTGAGTGT		
<i>OCT4</i>	Forward	GACAAGGAGAAGCTGGAG	54	JF898834
	Reverse	GCAAATTGTTCAAGGTCTTTC		
<i>NANOG</i>	Forward	GGGAAGGGTAATGAGTCCAA	56	DQ487022
	Reverse	AGCCTCCCTATCCCAGAAAA		
<i>SOX2</i>	Forward	CATGGCAATCAAAATGTCCA	54	DQ126150
	Reverse	AGACCACGGAGATGGTTTTG		
<i>CD 29</i>	Forward	CTTATTGGCCTTGCATTGCT	58	XM.005606848
	Reverse	TTCCTCGTACTTCGGATTG		
<i>CD 34</i>	Forward	CACTAAACCCTCTACATCATTTTCTCCTA	60	XM 001491596
	Reverse	GGCAGATACCTTGAGTCAATTCA		
<i>CD44</i>	Forward	ATCCTCACGTCCAACACCTC	58	NM_001085435.1
	Reverse	CTCGCCTTTCTTGGTGTAGC		
<i>HDAC2</i>	Forward	GACATATGAGACTGCAGTTGC	57	XM_004370 57
	Reverse	ACCTCCTTCTCCTTCATCCTC		
<i>DNMT1</i>	Forward	ATAAGTAAGATAGTGGTTGAGTTC	55	NM_182651
	Reverse	TTGAGCATACAAGGAGGAA		
<i>DNMT3A</i>	Forward	GACAAGAATGCCACCAAAAGC	55	AY271298
	Reverse	ATCCACCAAGACACAATGCG		
<i>STAT3</i>	Forward	GCGAAGAATCAAGCAGTTCC	60	DQ487026
	Reverse	CCAGGGCAGTAAGCATCTGT		
<i>IGF2</i>	Forward	CTTCAGCCGACCATCCAG	55	FJ032306
	Reverse	GGGGTGGCACAGTAAGTC		
<i>IGF2R</i>	Forward	GCAGATTTATTCTTCTCCAC	55	J03527
	Reverse	CACTCAAACCTCGTAGAAGCA		
<i>MNSOD</i>	Forward	GGGCCATATCAATCACAG	55	JN687584
	Reverse	GTCCCTGCTCCTTATTG		
<i>GLUT-1</i>	Forward	CCGTCTCTTCCTATCCAA	56	HQ434959
	Reverse	GGTCTTCTTGAATAGTGAGTT		
<i>BAX</i>	Forward	ATCCACCAAGAAGCTGAG	52	HE661581
	Reverse	CTGCGATCATCCTCTGTA		
<i>BCL2</i>	Forward	GGCCCCTGTTTGATTCT	56	U92434
	Reverse	CTTATGGCCCAGATAGGC		

Collection of oocytes

Ovaries from reproductive organs of apparently healthy adult buffaloes were collected from abattoir within 30 min of slaughter and 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). The aspirated 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 homogenous granular ooplasm were selected for in vitro maturation.

In vitro maturation (IVM)

The IVM of COCs was performed as described earlier by Sadeesh et al. (2014b). In brief, 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 immediately before use. The COCs were washed several times with IVM medium and groups 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 cytoplasm and hand-made cloning (HMC)

The recipient cytoplasm 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. 2014a). In brief, 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 (FAF) 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. Embryo production rate was examined under inverted microscope (Nikon Inc., Tokyo, Japan) to record the number of cleaved embryos at 48 h post-activation (h.p.a), percentage of embryos with 8- to 16-cells at 94–96 h.p.a and blastocyst formation at 168–192 h.p.a. Blastocysts were stained with Hoechst 33342 for 1 h and the total number of their nuclei was counted as described earlier by Saikhun et al. (2004).

In vitro embryo production through in vitro fertilization

In vitro maturation

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

Sperm capacitation

Spermatozoa were capacitated using Brackett and Oliphant medium (BO; Brackett and Oliphant 1975). Semen was obtained from the semen lab of Central Institute for Research on Buffaloes (Hisar, India). In all experiments, frozen semen from the same bull was used. Frozen semen from buffalo bulls stored in 0.25 ml straws was thawed in 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 around 0.5 ml of the washing BO medium and the sperm number was counted using hemocytometer and number of spermatozoa were adjusted to be 2×10^6 /ml.

In vitro fertilization procedure

For in vitro fertilization, 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 FAF-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 in vitro fertilization.

Embryo culture

The activated embryos were cultured using the same culture protocol as described for the NT embryos and the number of cleaved embryos, 8- to-16-cell and

blastocyst stages at 48 h post-insemination (h.p.i), 94–96 h.p.i and 168–192 h.p.i, respectively, were recorded. Blastocysts were stained with Hoechst 33342 for 1 h and the total number of their nuclei was counted as described earlier by Saikhun et al. (2004).

Gene expression analysis in embryos

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

Morphologically normal 8- to 16-cell and blastocyst stages NT and IVF embryos were separately treated using a Cell-to-cDNA kit (Ambion Inc, The RNA Company, Austin, TX) according to the manufacturers' protocol immediately after taking photographs. Ten–12 embryos at 8-to 16- cell stage and five to six embryos at blastocyst stage were analysed in each group in a trial ($n = 6$). The embryos were washed with 200 μ l ice cold PBS after which 50 μ l 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 μ l of the cell lysates (RNA), 4 μ l dNTP mix (2.5 mM each dNTP), 2 μ l random decamer were taken in 200 μ l PCR tube. The reaction mixture was mixed and incubated at 70 °C for 1 min to denature RNA for easier binding of primer in a thermal cycler, the tubes were cooled immediately on ice and the remaining reverse transcriptase reagents were added: 2 μ l 10X RT buffer, 1 μ l MMLV reverse transcriptase and 1 μ l RNase inhibitor. 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 μ l reaction to assess the gene expression of *OCT4*, *NANOG*, *HDAC2*, *DNMT1*, *DNMT3A*, *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 μ l volume reaction mixture containing 12.5 μ l of SYBR Green qPCR mastermix, 1 μ l of primer (10 pM each forward and reverse primer), 2 μ l of cDNA template and 9.5 μ l of nuclease free water. Samples not exposed to reverse transcriptase (RT) were used as negative controls. For PCR, samples were activated at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 45 s, then annealing at the specific primer annealing temperature (Table 1) for 30 s and extension at 72 °C for 30 s. 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 Δ CT 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 Δ CT as an arbitrary constant to subtract from all other Δ CT samples values. Fold changes in relative mRNA expression of the target genes were determined using the formula $2^{-\Delta\Delta CT}$.

Experimental design

The present study comprised of four experiments. In Experiment 1, isolation and characterization of buffalo FFs were evaluated. In Experiment 2, isolation and characterization of buffalo AMSCs for the developmental pluripotency by pluripotency, mesenchymal cell surface makers and multilineage differentiation were evaluated. In Experiment 3, the suitability of FFs and AMSCs as nuclear donors was assessed by comparing the developmental rate and total cell number. In experiment 4, the gene expression patterns in NT embryos reconstructed with FFs and AMSCs were examined. Embryos produced by in vitro fertilization were used as control.

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.

Results

Isolation, culture and characterization of FFs

The FFs derived from buffalo fetal skin started emerging and anchoring to cell culture flasks within 24 h after placing the minced tissue on re-calcified plasma drops. Majority of cells adhered to the surface of culture flasks. The attached cells expanded with spindle-shaped morphology resulting in primary cultures with the first confluency time of 4–5 days. Representative photographs of cultured FFs are shown in Fig. 1a–d. Two biological (one at the early passage and one at the later cell culture passage) and three technical replicates were analysed. For cell type determination, the samples were examined using fluorescence microscopy after immunofluorescent staining. Results of the fetal fibroblast cell lines were positive for vimentin and negative for keratin (Fig. 2). Growth curve of these cells showed a typical “S” shape

(Fig. 3a). An adaptive phase was apparent after cells were seeded. This phase lasted about 2 days. The cells then entered a platform period, after which they showed a logarithmic growth period lasting 4 or 5 days. The population doubling time (PDT) was 41.4 h (Fig. 3b).

Isolation, culture and characterization of AMSCs

The cells isolated from amnion were also cultured in the same condition as those isolated from fetal skin. The media were changed every 3–4 days. The initial growth of AMSC cultures at passage 0 (P0) consisted of two different heterogeneous populations; one with fibroblast-like morphology and the other with epithelial-like morphology. It was observed that growth of epithelial-like cells was slower than that of fibroblast-like cells. Upon trypsinisation and sub-cultivation, the epithelial like population disappeared from the culture and could no longer be found in subsequent passages. Representative photographs of cultured AMSCs are shown in Fig. 1e–h. The passaged cells exhibited higher growth rate, reaching a 95–100 % confluence 10 days after plating. As described in studies performed in other species, the cells grew with regular pace until passage 10 and then they started to slow down and growth was arrested at passage 15. Reduction of growth was

Fig. 1 Isolation and culture of FFs and AMSCs. **a** Primary explants outgrowth from fetal explant, **b** sub-cultured FFs having 40–50 % confluence, **c** sub-cultured fetal fibroblast cells having 70–80 % confluence, **d** confluent monolayer of sub-cultured cells of fetal fibroblast; **e** heterogeneous populations of

AMSCs with fibroblastic and epithelioid morphologies at passage 0, **f** disappearance of the epithelioid population and appearance of fibroblast-like cells upon trypsinisation and passaging, **g** homogeneous population of AMSCs, **h** homogeneous population of AMSCs with cluster (200 μ m)

Fig. 2 Expression of cell-specific marker in somatic cells derived from fetal skin of buffalo. **a** Immunofluorescent staining of vimentin (red nucleus, green vimentin), **b** immunofluorescent staining of keratin (50 μ m). (Color figure online)

Fig. 3 Cell proliferation rate (**a**) and population doubling time (**b**) of FFs and AMSCs

accompanied by increasing cellular dimension and by changing morphology from spindle-shaped to flat suggesting senescence. Two biological (one at the early passage and one at the later cell culture passage) and three technical replicates were analysed. The growth curve of AMSCs (Fig. 3a) had the following characteristics: (1) in the first 2 days after inoculation the cells adhered to the tissue culture plates, (2) on day 3 the cells entered the logarithmic growth stage, (3) peak growth was on day 10, and (4) the population doubling time (PDT) was found to be 39.5 h (Fig. 3b).

Characterisation of AMSC by alkaline phosphatase staining

Cell monolayer formed by buffalo AMSC showed alkaline phosphatase activity at different passages. The cells were found to stain positive for AP. The

AMSCs showed ES cell-like cell properties by AP staining. The cultured AMSCs were stained red and were considered positive for AP expression (Fig. 4a).

Characterization of AMSCs for pluripotency and mesenchymal stem cell surface markers

The expression of pluripotency markers (*OCT4*, *NANOG* and *SOX2*), mesenchymal stem cell surface markers (*CD29* and *CD44*) and haematopoietic marker (*CD34*) genes was studied to characterize the AMSCs for stemness property. Agarose gel electrophoresis for analysis of RT-PCR products revealed PCR amplicons of 76, 182, 215, 169, 165 bp, respectively, of *OCT4*, *NANOG*, *SOX2*, *CD29* and *CD44* genes in buffalo AMSCs with *GAPDH* (131 bp) as housekeeping gene (Fig. 4b), which indicate the mesenchymal stemness of the cultured cells. The

Fig. 4 Characterization of AMSCs. **a** Alkaline phosphatase staining showing red colored AP positive cells (100 μ m), **b** RT-PCR analysis of pluripotent (*OCT4*, *NANOG* and *SOX2*),

mesenchymal (*CD29* and *CD44*), and hematopoietic (*CD34*) specific gene expression on buffalo AMSCs. (Color figure online)

expression of the haematopoietic stem cell marker *CD34* was negative in AMSCs (Fig. 4b).

In vitro differentiation potential of AMSCs

When AMSCs were cultured under adipogenic conditions, they differentiated into adipocytes and exhibited high intensity of oil red O stain in the cytoplasm of cells on the 21st day of post-induction, signifying the presence of lipid globules (Fig. 5a). The chondrogenic potential of AMSCs was evaluated by in vitro culture of these cells in a specific serum-free chondrogenic medium. The accumulation of sulfated proteoglycans was visualized by alcian blue staining on the 21st day of post-induction (Fig. 5b). When AMSCs were cultured in osteogenic differentiation medium, the cells started changing morphology after 8–9 days of incubation. The cells were stained with alizarin red on the 21st day of culture in osteogenic condition. The positive expression of alizarin red confirmed the presence of calcium deposits in differentiated cells (Fig. 5c). Cells maintained in regular control medium did not stain positively in any of the groups.

Post-thaw cell viability

Viability of FFs and AMSCs was 97.42 and 96.27 % before cryopreservation and 86.48 and 84.53 % post thaw, as assessed by trypan blue dye exclusion method. The culture behaviour and morphology of freeze–thawed cells were similar to those of fresh cells

Fig. 5 In vitro multipotent differentiation potential of AMSCs. **a** Oil red O positive adipogenic differentiated cells, **b** alcian blue positive chondrogenic differentiated cells, **c** alizarin red positive osteogenic differentiated cells (100 μ m)

In vitro developmental ability of in vitro fertilized (IVF) and NT embryos from FFs and AMSCs

The SCNT and IVF embryonic development is presented in Fig. 6. The cleavage rate was significantly ($P < 0.05$) higher in IVF (82.6 ± 8.2 %) than in NT embryos from FF and AMSC (64.6 ± 1.3 and 72.3 ± 2.2 %, respectively). However, blastocyst

rates in IVF and NT embryos derived from AMSCs did not differ (30.6 ± 2.7 and 28.9 ± 3.1 %, respectively) but were significantly ($P < 0.05$) higher than NT embryos from FFs (19.5 ± 1.8 %). Total cell numbers did not show significant ($P > 0.05$) differences between IVF and NT embryos derived from AMSCs (186.7 ± 4.2 , 171.2 ± 3.8 , respectively) but were significantly ($P < 0.05$) higher than from FFs (151.3 ± 4.1) (Table 2).

Comparison of gene expression patterns in embryos derived from different origin

The relative abundance (RA) of the gene transcripts at the 8- to 16-cell, and blastocyst stages in IVF and NT

embryos is shown in Fig. 7. The RA of *OCT4* transcript at the 8- to 16-cell and blastocyst stages, did not vary between FF and AMSC-NT embryos but was significantly ($P < 0.05$) lower than in IVF embryos. The RA of *NANOG* transcript at the 8- to 16-cell and blastocyst stages, did not vary between FF and AMSC-NT embryos but was significantly ($P < 0.05$) higher than in IVF embryos. For *STAT3* transcript, differences between IVF and NT embryos were significant ($P < 0.05$) at the blastocyst stage, where the levels were higher in AMSC-NT embryos. The *DNMT1* levels at the 8- to 16-cell were significantly ($P < 0.05$) higher in FF-NT embryos followed by AMSC-NT and IVF embryos, but there were no differences at the blastocyst stage. The RA of

Fig. 6 In vitro development of buffalo IVF (**a1–a3**) and cloned embryos derived from fetal fibroblasts (**b1–b3**) and AMSCs (**c1–c3**). **a1, b1, c1** 8-cell stage embryo, **a2, b2, c2** 16-cell stage embryo, **a3, b3, c3** blastocyst (50 μ m)

Table 2 In vitro developmental potential of IVF and NT buffalo embryos

Group	No. of embryos cultured	Cleaved embryos (%)	Blastocysts (%)	Total cell numbers
FFs	102	64.6 ± 1.3 ^a	19.5 ± 1.8 ^a	151.3 ± 4.1 ^a
AMSCs	103	72.3 ± 2.2 ^a	28.9 ± 3.1 ^b	171.2 ± 3.8 ^b
IVF	108	82.6 ± 8.2 ^b	30.6 ± 2.7 ^b	186.7 ± 4.2 ^b

Figures quoted as percent mean ± SEM

Cleavage, blastocyst and total cell number values having different superscripts in the same column differ significantly ($P < 0.05$)

Fig. 7 Relative expression profile of developmentally important genes in IVF and NT (FF-NT and AMSC-NT) embryos at 8-to 16-cell and blastocyst stages. Values are presented as mean ± SEM. Bars without a common superscript differ significantly ($P < 0.05$)

DNMT3A was high at the 8-to 16-cell stage in FF-NT embryos, where the differences were non significant ($P > 0.05$) between IVF and AMSC-NT embryos.

Differences between IVF and AMSC-NT embryos were non significant ($P > 0.05$) at the blastocyst stage, for which the levels were significantly ($P < 0.05$)

lower in FF-NT embryos. For *HDAC2* transcript, at the 8- to 16-cell stage, for which no significant ($P > 0.05$) variation was observed between NT embryos, was significantly ($P < 0.05$) higher than in IVF embryos. No differences ($P > 0.05$) were found between IVF and AMSC-NT embryos at the blastocyst stage, whereas lower levels were detected in FF-NT embryos. The *IGF2* transcript level was significantly ($P < 0.05$) higher at the 8- to 16-cell and blastocyst stages in IVF and FF-NT than in AMSC-NT embryos. The RA of *IGF2R* was high at the 8- to 16-cell stage in FF-NT embryos, whereas the differences were non significant ($P > 0.05$) between IVF and AMSC-NT: no significant ($P > 0.05$) differences were detected between IVF and NT embryos at the blastocyst stage. Significantly ($P > 0.05$) higher RA of *BAX* was observed in FF-NT embryos, followed by AMSC-NT and IVF embryos in all stages. Transcript abundance of *BCL2* was significantly ($P > 0.05$) higher in IVF embryos at 8- to 16-cell stage as compared to NT. No significant ($P > 0.05$) differences in the RA of *BCL2* were observed between AMSC-NT and IVF embryos at the blastocyst stage, whereas lower levels were detected in FF-NT embryos. The RA of *MnSOD* was significantly ($P < 0.05$) higher in IVF embryos at the 8- to 16-cell and blastocyst stages as compared to NT. No significant ($P > 0.05$) differences in RA of *GLUT1* transcript were observed between IVF and AMSC-NT embryos at the 8- to 16-cell stage, whereas higher levels were detected in FF-NT embryos. Differences between IVF and AMSC-NT embryos were non significant ($P > 0.05$) at the blastocyst stage, whereas the levels were lower in FF-NT embryos.

Discussion

For successful SCNT, proper nuclear reprogramming in somatic cell genome by the recipient cytoplasm is a must. The source of biological material has a great impact on the outcome of cloning experiments (Vajta 2007). As far as cloning of buffalo is concerned, reconstruction of embryos remains one of the most tricky and challenging part of NT procedures, although NT has been performed in certain laboratories (Shah et al. 2008; Saha et al. 2012; Sadeesh et al. 2014a, b). The success rate of obtaining live offspring from cloned buffalo embryos is still very low.

The State of the donor cell is one of the most important factors for cloning efficiency. Developmental abnormalities in cloned mammals are mainly due to abnormal epigenetic reprogramming of the donor genome (Li et al. 2003). The competence of reprogramming by NT varies significantly for the different types of cells used. Yang et al. (2007) reported that the success of nuclear reprogramming decreases as donor cells become more differentiated. It has been hypothesized that the genome of undifferentiated cells, such as stem cells, may be more easily reprogrammed by the recipient oocyte. The relationship between donor cell differentiation status and NT success has been demonstrated in mice, with NT embryos derived from ES cells (Rideout et al. 2000; Eggan et al. 2001) showing significantly enhanced survival to term compared with those derived from somatic cell nuclei (Wakayama and Yanagimachi 1999). However, Hochedlinger and Jaenisch (2002) obtained cloned mice from terminally differentiated, mature T and B-cells using a two-step method, but they never succeeded with a simple NT. Cloned mice were successfully obtained from the nucleus of natural killer T cell lymphocytes, a lymphocyte population in the same hematopoietic lineage, but not from peripheral T cells (Inoue et al. 2005). Contrastingly, in vitro development of hematopoietic stem cells (HSC) cloned embryos was very poor and the birth rates per transfer were no better than those of clones from other somatic cell types such as cumulus, immature sertoli and fibroblast cells (Inoue et al. 2006) suggesting that the genome of HSC may have a lower genomic plasticity, at least in terms of the ability to be reprogrammed in the metaphase II cytoplasm after NT. This suggests that a cell in an undifferentiated state may be a suitable donor cell in animal cloning. Although ES cells may be successful for NT in mice, this process is limited in other species where definitive ES cells have not been established. Thus investigating other somatic stem cells used in NT is necessary. The developmental potential of the cloned embryos derived from amniotic fluid derived stem cells being higher than that of the cloned embryos derived from adult fibroblasts has previously been reported in pig (Zhao and Zheng 2010) and buffalo (Sadeesh et al. 2014a). The possibility of using AMSCs as donors was explored in the present study in view of the very low overall cloning efficiency in buffalo.

After culturing for a few passages, purified fibroblasts were confirmed by immunofluorescent staining for vimentin and lack of keratin. This result is in agreement with Sadeesh et al. (2014a). They reported that the somatic cells derived from the skin of buffalo were found to be of fibroblast origin as these expressed vimentin but not keratin. In the present work, fetal fibroblasts of skin tissue were successfully established using adherent culture methodology. The growth curves of these cells showed a typical “S” shape and the population doubling time was 41.4 h. Cell viability before freezing and after thawing was above 85 % for these cells which indicate that fetal fibroblasts have a greater tolerance to trypsin digestion and liquid nitrogen cryogenic preservation.

It is a well-known fact that mixed population of cells cultured from amniotic membrane in many species such as human, ovine, chicken, rat, equine and canine also displayed heterogeneous cell population (Marcus et al. 2008; Mauro et al. 2010; Bacenkova et al. 2011; Coli et al. 2011; Gao et al. 2012; Park et al. 2012). In agreement with these reports, it was observed that cultured cells exhibited both spindle-shaped fibroblasts and polygonal shaped epithelial cell-like morphology in vitro. In addition, it was found that growth was rapid at early passages up to 5–6 passages. A consistent level of cell proliferation is a distinct characteristic of stem cells. However, in the present experiments, cells stopped proliferating after 15 passages thus indicating non-optimized culture condition for these cells to proliferate continuously in vitro. The AMSC had a doubling time of 39.5 h which was comparable to the doubling time of bovine AMSCs (Corradetti et al. 2013). Another parameter that indicated the high proliferative property of the isolated cells was their growth curve characteristics. According to this calibrated curve plotted for AMSC, there was a short lag phase implying that the cells rapidly recovered from the damage which occurred during detachment by enzymatic treatment. A strong positive expression of AP staining was demonstrated in AMSCs. AP is a stem cell membrane marker, and elevated expression of this enzyme is associated with the undifferentiated pluripotent stem cell state. Most of the pluripotent stem cells, like ES and embryonal carcinoma cells, express AP activity (Shamblott et al. 1998; Hua et al. 2009).

Using species-specific primers, PCR amplicons of 76, 182 and 215 bp were observed for *OCT4*, *NANOG*

and *SOX2*, respectively, in buffalo AMSCs. These results indicate that these cells were in an undifferentiated state. Expression of these pluripotency marker genes has previously been reported in amnion-derived cells of various mammalian species including rat (Marcus et al. 2008), canine (Park et al. 2012) and chicken (Gao et al. 2012). In addition, reverse transcriptase PCR results indicated that AMSCs expressed the mesenchymal stem cells markers *CD44* and *CD29*, but not haematopoietic surface markers such as *CD34* as reported earlier in bovine (Corradetti et al. 2013). *CD29* is an integrin unit involved in cell adhesion and *CD44* is a multicellular and multifunctional marker involved in cell proliferation. The expression pattern of marker genes indicated that buffalo MSCs have an expression pattern which is characteristic of typical MSCs. Fibroblast-like appearing adherent MSCs isolated from buffalo amnion were successfully induced to multipotential differentiation into adipogenic, chondrogenic, and osteogenic lineages under specific culture conditions. The morphological features of MSCs were similar to the earlier observations of Corradetti et al. (2013). It is widely accepted that multiple cell types can be derived by culturing AMSCs under appropriate conditions. Several laboratories have reported neural (Miki et al. 2005; Ilancheran et al. 2007; Tamagawa et al. 2008), hepatic (Miki et al. 2005; Ilancheran et al. 2007; Tamagawa et al. 2007), cardiac (Ilancheran et al. 2007; Tsuji et al. 2010), osteogenic (Ilancheran et al. 2007; Bilic et al. 2008), chondrogenic (Wang et al. 2010) and adipogenic (Ilancheran et al. 2007) differentiation of AMSCs. The multipotent developmental characteristics of AMSCs may increase buffalo cloning efficiency, if the undifferentiated state of the donor cells affects the success rate as observed with ES cell donors. However, there are no previously published reports on production of cloned embryos using buffalo AMSCs.

To our knowledge this is the first comparative study on the effects of FFs and AMSCs on embryo cloning in buffaloes. The results suggest that FFs and AMSCs can be reprogrammed via HMC to support the development of the resultant embryos to the blastocyst stage in buffalo. However, cleavage, blastocyst formation and mean cell numbers per blastocyst for cloned buffalo embryos derived from AMSCs were significantly ($P < 0.05$) higher than those derived from FFs. This reveals that AMSCs were not similar to

FFs in terms of cell cycle which may be because of the state of donor cells. Thus, these results support the notion that the source of the donor cells may be one of the most important factors in determining the success of NT (Miyoshi et al. 2003; Powell et al. 2004). In general, due to the rapid growth and the potential for multiple cell divisions, FFs have been commonly used as nuclear donors in buffalo cloning. The blastocyst rate of buffalo embryos cloned with FFs is below the level of 25 % (Shah et al. 2009). These data are almost similar with our data showing that 19.5 % of cloned embryos with FFs develop to blastocysts. In the present study, the highest rate of blastocysts formation of the embryos cloned with undifferentiated MSCs as nuclear donor may increase cloning efficiency. Furthermore, the higher total cell number of embryos cloned with undifferentiated MSCs are of higher quality than embryos cloned with differentiated cells. This is consistent with porcine MSCs. MSCs have been successfully established, and these cells have been further employed as nuclear donors and significant differences in cleavage and blastocyst rates were found using MSCs as donor cell in porcine as compared to FFs (Jin et al. 2007). This indicates that the phenomenon of epigenetic reprogramming can be made more efficient by the use of undifferentiated MSCs as these cells represent an intermediate stage between pluripotent ES cell stage and lineage restricted adult stem cells.

Qualitative or morphological assessment alone is not enough in providing an accurate and efficient estimation of embryo quality and embryonic developmental potential (Wrenzycki et al. 2004). To the best of our knowledge, this is the first report comparing mRNA expression patterns of development-related genes among pre-implantation NT embryos from FFs and AMSCs in buffalo species. 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 maternal to zygotic transition (MZT) between NT and IVF embryos. 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. 2006; 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*); metabolism (*GLUT1*).

Similar to the present study levels of *OCT4* expression has previously been reported in bovine (Hall et al. 2005; Amarnath et al. 2007; Long et al. 2007; Zhou et al. 2008) and buffalo (Sadeesh et al. 2014b) between cloned and IVF embryos. However, Daniels et al. (2000), using bovine granulosa cells as donors, found dissimilar results. The present study demonstrated that *OCT4* expression in NT embryos at the blastocyst stage was significantly ($P < 0.05$) 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. The 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 downregulation of *OCT4* gene is considered to be a candidate marker for the low potential of NT embryos to develop into young ones (Kato and Tsunoda 2010). These 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, considering 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). These 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 hypothesize 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, a higher abundance of *STAT3* was observed in NT embryos derived from AMSCs than

from FFs. Up-regulation of *STAT3* may influence cell proliferation (Zhu et al. 2004) and, interestingly, the results of the present experiments showed an increase in the number of cells at the blastocyst stage in IVF and also to some extent in AMSC-NT embryos in contrast to *STAT3* down-regulation and low cell number in FF-NT blastocysts.

Earlier studies have revealed species-dependent expression patterns of DNA methyltransferases (*DNMTs*) genes in mammalian oocytes and pre-implantation embryos (Vassena et al. 2005), and also an association between incomplete DNA methylation and the lack of NT success in mammals (Dean et al. 2001; Bortvin et al. 2003; Chung et al. 2003). Severely reduced transcript levels of *DNMT1* have been reported in cloned than in IVF or parthenote bovine embryos by Golding et al. (2011). The results of the present study revealed that as compared to IVF embryos the RA of *DNMT1* transcript was significantly ($P < 0.05$) increased in NT embryos. This 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 in vitro development for SCNT embryos (Suteevun et al. 2006). These abnormal levels of embryonic methylation may be connected with reduced developmental potential of NT embryos. Expression of *DNMT3A* in AMSC-NT and IVF blastocysts was similar, whereas it was lower in FF-NT blastocyst. This suggests that differential *DNMT3A* mRNA levels in FF-NT embryos have serious implications for the pre-implantation development and result in the lower number of blastocyst formation of FF derived 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 FF and AMSC-derived NT embryos using IVF embryos as controls, before meaningful conclusions can be drawn in this species.

It is known that acetylation and deacetylation of the lysines in the tails of the core histones are controlled by the actions of two families of enzymes, histone acetyltransferases (*HAT*'s) and histone deacetylases

(*HDACs*). In general, histone acetylation is thought to facilitate transcription, and this effect is reversed by deacetylation which correlates with gene repression. Their dynamic balance regulates gene transcription and gene expression of eukaryotes at the DNA level. Some anomalous expression of *HDACs* has been observed in SCNT embryos (Suteevun et al. 2006; Li et al. 2008; Imsoonthornruksa et al. 2010; Lee et al. 2010). In this study, at the blastocyst stage *HDAC2* expression was similar for AMSC-NT embryos and IVF embryos, whereas a lower level was detected in FF-NT embryos. This might be an indication of failed reprogramming of inefficient FF nuclei after transfer in 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 the reason for the differential expression of *HDACs* in cloned blastocyst produced from different cell lines in these studies. Taken together, these results indicate that the donor genome was not reprogrammed to switch over the pattern of gene expression at the appropriate time from the somatic to the embryonic type following NT.

The *IGF2* and *IGF2R* genes are among the best studied imprinted genes involved in fetal growth regulation and are essential for normal development. The *IGF2* ligand is imprinted when inherited maternally and *IGF2R* is imprinted when inherited paternally (Latham et al. 1994). In the experiment, the expression of *IGF2* and *IGF2R* transcripts was significantly ($P < 0.05$) increased in IVF embryos as compared to NT counterparts. Alteration in mRNA expression of *IGF-2* between NT- and IVF-derived blastocysts can be associated with inappropriate genomic imprinting because of differences in embryo production procedure (Wrenzycki et al. 2001). 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*. Furthermore, 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 which are regulated by genomic imprinting (Feil 2001). This is 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 rare.

The pro-apoptotic *BAX* gene is used in the analysis of apoptosis in oocytes and embryos (Yang and Rajamahendran 2002; Opiela et al. 2008). The data from the current study indicate that *BAX* expression was significantly ($P < 0.05$) higher in NT embryos from FFs than in embryos from AMSCs and IVF, suggesting that over expression of the *BAX* gene, the cellular apoptotic pathway takes place (Yang and Rajamahendran 2002; Opiela et al. 2008). This non-regulation of *BAX* mRNA expression could be a cause for the low quality of FF-NT embryos. *BCL2* is considered to be an anti-apoptotic gene, and its expression level should be low when apoptotic incidence is high. The results of the present study also revealed that the expression level of *BCL2* in FF-NT embryos was significantly ($P < 0.05$) lower than that of IVF and AMSC embryos at the blastocyst stage. Yang and Rajamahendran (2002) demonstrated that good quality embryos have a greater concentration of *BCL2* protein than that of *BAX* protein, whereas there is more *BAX* than *BCL2* in lower quality embryos. The higher apoptotic incidence in NT embryos than in the IVF group may be attributed to the in vitro micromanipulation (McElroy et al. 2008). The in vitro micromanipulation could cause cellular mechanical damage to some extent, resulting in higher apoptotic rate of buffalo NT-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).

Reactive oxygen species (ROS) are indicated as being one of the main factors responsible for the low production and poor quality of embryos produced in vitro (Takahashi et al. 2000). It has also been observed that physiological levels of ROS are necessary for normal regulation of cell growth and development (Hancock et al. 2001). The *MnSOD* is an important enzyme involved in the protection against ROS. The aberrant expression of this gene at the 8- to 16-cell, and blastocyst stages in NT embryos suggests that NT embryos fail to establish an appropriate embryonic *MnSOD* expression pattern. Our results are contradictory to Kim et al. (2012). They reported no differences in the expression levels of *MnSOD* between bovine NT and IVF embryos. This

divergence among experiments may be due to different protocols used to produce and culture embryos and due to different methods to assess gene expression profile.

The cellular glucose incorporation into embryonic cells is mediated by *GLUT1* and its expression has been detected during the entire pre-implantation period (Lequarre et al. 1997; Bertolini et al. 2002). 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 suggest that viable embryos have higher *GLUT1* expression. The decrease in the relative expression of *GLUT1* in FF-NT blastocyst supports the notion that FF-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). Therefore, it is possible that the low level of *GLUT1* expression observed in the present study in FF-NT embryos is related to higher levels of apoptotic incidence in these embryos at the blastocyst stage.

In conclusion, it was demonstrated in the present study that buffalo AMSCs exhibit high proliferation rates and multilineage differentiation potential. The NT embryos reconstructed with AMSCs show enhanced developmental potential as compared to those reconstructed with FFs, with a significantly higher proportion of embryos reaching the blastocyst stage. High total cell number was associated with NT embryos derived from AMSCs. 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; Alvarez et al. 2010). In these studies, the RA of target transcripts in AMSC-NT embryos more closely followed that of the IVF derived embryos as compared with FF-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 the results of an enhanced pre-implantation development with increased cell number in AMSC-NT embryos as compared to the FF-NT counterparts. The low developmental

competence of pre-implantation FF-NT embryos may, at least in part, be due to inadequate reprogramming leading to defects in early embryonic gene expression. Further, it is 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 FF-NT embryos when compared with AMSC-NT and IVF embryos. Therefore, these results suggest that the developmental potential of the AMSC-NT embryos was higher than that of the cloned embryos derived from FF which may be due to the fact that AMSC with relatively undifferentiated genome reprogrammed more efficiently to reactivate expression of early embryonic genes.

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References

- Alvarez LR, 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
- 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
- Bacenkova D, Rosocha J, Tothová T, Rosocha L, Sarisky M (2011) Isolation and basic characterization of human term amnion and chorion mesenchymal stromal cells. *Cytotherapy* 13:1047–1056
- Bertolini A, Beam SW, Shim H, Bertolini LR, Al Moyer, Famula TR, Anderson GB (2002) Growth, development and gene expression by in vivo and in vitro-produced day 7 and 16 bovine embryos. *Mol Reprod Dev* 63:318–328
- 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
- Bilic G, Zeisberger SM, Mallik AS, Zimmermann R, Zisch AH (2008) Comparative characterization of cultured human term amnion epithelial and mesenchymal stromal cells for application in cell therapy. *Cell Transplant* 17:955–968
- Bortvin A, Eggan K, Skaletsky H, Akutsu H, Berry DL, Yanagimachi R, Page DC, Jaenisch R (2003) Incomplete reactivation of *OCT4*-related genes in mouse embryos cloned from somatic nuclei. *Development* 130:1673–1680
- Bracket BG, Oliphant G (1975) Capacitation of rabbit spermatozoa in vitro. *Biol Reprod* 12:260–274
- 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
- Chung YG, Ratnam S, Chaillet JR, Latham KE (2003) Abnormal regulation of DNA methyltransferase expression in cloned mouse embryos. *Biol Reprod* 69:146–153
- Coli A, Nocchi F, Lamanna R, Iorio M, Lapi S, Urciuoli P, Scatena F, Giannessi E, Stornelli MR, Passeri S (2011) Isolation and characterization of equine amnion mesenchymal stem cells. *Cell Biol Int Rep* 18:e00011
- Colleoni S, Donofrio G, Lagutina I, Duchi R, Galli C, Lazzari G (2005) Establishment, differentiation, electroporation, viral transduction and nuclear transfer of bovine and porcine mesenchymal stem cells. *Cloning Stem Cells* 7:154–166
- Corradetti B, Meucci A, Bizzaro D, Cremonesi F, Consiglio AL (2013) Mesenchymal stem cells from amnion and amniotic fluid in the bovine. *Reproduction* 145:391–400
- Daniels R, Hall V, Trounson AO (2000) Analysis of gene transcription in bovine nuclear transfer embryos reconstructed with granulosa cell nuclei. *Biol Rep* 63:1034–1040
- De Coppi P, Jr Georg Bartsch, Siddiqui MM, Xu T, Santos CC, Perin L, Mostoslavsky G, Serre AC, Snyder EV, Yoo JJ, Furth ME, Soker S, Atala A (2007) Isolation of amniotic stem cell lines with potential for therapy. *Nat Biotechnol* 25:100–106
- Dean W, Santos F, Stojkovic M, Zakhartchenko V, Walter J, Wolf E, Reik W (2001) Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. *Proc Natl Acad Sci USA* 98:13734–13738
- Eggan K, Akutsu H, Loring J, Grusby LJ, Klemm M, Rideout WM, Yanagimachi R, Jaenisch R (2001) Hybrid vigor, fetal overgrowth and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *Proc Natl Acad Sci USA* 98:6209–6214
- 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
- Gao Y, Pu Y, Wang D, Zhang W, Guan W, Ma Y (2012) Isolation and biological characterization of chicken amnion epithelial cells. *Eur J Histochem* 56:e33
- 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
- Golding MC, Williamson GL, Stroud TK, Westhusin ME, Long CR (2011) Examination of DNA methyltransferase expression in cloned embryos reveals an essential role for *DNMT1* in bovine development. *Mol Reprod Dev* 78:306–317

- Gucciardo L, Lories R, Ochsenbein-Kölble N, Done' E, Zwijsen A, Deprest J (2009) Fetal mesenchymal stem cells: isolation, properties and potential use in perinatology and regenerative medicine. *BJOG* 116:166–172
- Guest DJ, Smith MR, Allen WR (2010) Equine embryonic stem-like cells and mesenchymal stromal cells have different survival rates and migration patterns following their injection into damaged superficial digital flexor tendon. *Equine Vet J* 42:636–642
- Guillot PV, Gotherstrom C, Chan J, Kurata H, Fisk NM (2007) Human first-trimester fetal MSC express pluripotency markers and grow faster and have longer telomeres than adult MSC. *Stem Cells* 25:646–654
- Haig D, Graham C (1991) Genomic imprinting and the strange case of the insulin-like growth factor receptor. *Cell* 64:1045–1046
- Hall VJ, Ruddock NT, French AJ (2005) Expression profiling of genes crucial for placental and pre-implantation development in bovine in vivo, in vitro, and nuclear transfer blastocysts. *Mol Reprod Dev* 72:16–24
- Hancock JT, Desikan R, Neill SJ (2001) Role of reactive oxygen species in cell signalling pathways. *Biochem Soc Trans* 29:345–350
- 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
- Hochedlinger K, Jaenisch R (2002) Monoclonal mice generated by nuclear transfer from mature B and T donor cells. *Nature* 415:1035–1038
- Hua J, Yu H, Liu S, Dou Z, Sun Y, Jing X, Yang C, Lei A, Wang H, Gao Z (2009) Derivation and characterization of human embryonic germ cells: serum free culture and differentiation potential. *Reprod Biomed Online* 19:238–249
- Ilancheran S, Michalska A, Peh G, Wallace EM, Pera M, Manuelpillai U (2007) Stem cells derived from human fetal membranes display multilineage differentiation potential. *Biol Reprod* 77:577–588
- Imsoonthornruksa S, Lorthongpanich C, Sangmalee A, Sriratana K, Laowtammathron C, Tunwattana W, Somsa W, Ketudat-Cairns M, Parnpai R (2010) Abnormalities in the transcription of reprogramming genes related to global epigenetic events of cloned endangered felid embryos. *Reprod Fertil Dev* 22:613–624
- In't Anker PS, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE, Kanhai HH (2004) Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. *Stem Cells* 22:1338–1345
- Inoue K, Wakao H, Ogonuki N, Miki H, Seino K, Nambu-Wakao R, Noda S, Miyoshi H, Koseki H, Taniguchi M, Ogura A (2005) Generation of cloned mice by direct nuclear transfer from natural killer T cells. *Curr Biol* 15:1114–1118
- Inoue K, Ogonuki N, Miki H, Hirose M, Noda S, Kim JM, Aoki F, Miyoshi H, Ogura A (2006) Inefficient reprogramming of the hematopoietic stem cell genome following nuclear transfer. *J Cell Sci* 119:1985–1991
- Jin H, Kumar BM, Kim JG, Song HJ, Jeong YJ, Cho SK, Balasubramanian S, Choe SY, Rho GJ (2007) Enhanced development of porcine embryos cloned from bone marrow mesenchymal stem cells. *Int J Dev Biol* 51:85–90
- 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
- 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
- Kumar BM, Jin HF, Kim JG, Ock SA, Hong Y, Balasubramanian S, Choe SY, Rho GJ (2007) Differential gene expression patterns in porcine nuclear transfer embryos reconstructed with fetal fibroblasts and mesenchymal stem cells. *Dev Dyn* 236:435–446
- Latham K, Doherty AS, Scott CD, Schultz RM (1994) *IGF2R* and *IGF2* gene expression in androgenetic, gynogenetic and parthenogenetic pre-implantation mouse embryos: absence of regulation by genomic imprinting. *Genes Dev* 8:290–299
- Lee SL, Kang EJ, Maeng GH, Kim MJ, Park JK, Kim TS, Hyun SH, Lee ES, Rho GJ (2010) Developmental ability of miniature pig embryos cloned with mesenchymal stem cells. *J Reprod Dev* 56:256–262
- Lequarre AS, Grisart B, Moreau B, Schuurbiens N, Massip A, Dessy F (1997) Glucose metabolism during bovine pre-implantation development: analysis of gene expression in single oocytes and embryos. *Mol Reprod Dev* 48:216–226
- Li X, Li Z, Jouneau A, Zhou Q, Renard JP (2003) Nuclear transfer: progress and quandaries. *Reprod Biol Endocrinol* 1:84
- 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 (2006) Analysis of development related gene expression in cloned bovine blastocysts with different developmental potential. *Cloning Stem Cells* 8:41–50
- 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
- Liu C, Guo Y, Guanb W, Mab Y, Zhang H, Tang X (2008) Establishment and biological characteristics of Luxi cattle fibroblast bank. *Tissue Cell* 40:417–424
- Long JE, Cai X, He LQ (2007) Gene profiling of cattle blastocysts derived from nuclear transfer, in vitro fertilization and in vivo development based on cDNA library. *Anim Reprod Sci* 100:243–256
- Marcus AJ, Coyne TM, Rauch J, Woodbury D, Black IB (2008) Isolation, characterization, and differentiation of stem cells derived from the rat amniotic membrane. *Differentiation* 76:130–144
- Mauro A, Turriani M, Ioannoni A, Russo V, Martelli A, Giacinto OD, Nardinocchi D, Berardinelli P (2010) Isolation, characterization, and in vitro differentiation of ovine amniotic stem cells. *Vet Res Commun* 34:25–28
- 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
- Miki T, Lehmann T, Cai H, Stolz DB, Strom SC (2005) Stem cell characteristics of amniotic epithelial cells. *Stem Cells* 23:1549–1559
- Miyoshi K, Ruzicidlo SJ, Pratt SL, Stice SL (2003) Improvements in cloning efficiencies may be possible by increasing uniformity in recipient oocytes and donor cells. *Biol Reprod* 68:1079–1086
- 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, 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
- 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
- Paris DB, Stout TA (2010) Equine embryos and embryonic stem cells: defining reliable markers of pluripotency. *Theriogenology* 74:516–524
- Park SB, Seo MS, Kim HS, Kang KS (2012) Isolation and characterization of canine amniotic membrane-derived multipotent stem cells. *PLoS ONE* 7:e44693
- Powell AM, Talbot NC, Wells KD, Kerr DE, Pursel VG, Wall RJ (2004) Cell donor influences success of producing cattle by somatic cell nuclear transfer. *Biol Reprod* 71:210–216
- Rideout WM, Wakayama T, Wutz A, Eggan K, Jackson GL, Dausman J, Yanagimachi R, Jaenisch R (2000) Generation of mice from wild type and targeted ES cells by nuclear cloning. *Nat Genet* 24:109–110
- Rutigliano L, Corradetti B, Valentini L, Bizzaro D, Meucci A, Cremonesi F, Lange-Consiglio A (2013) Molecular characterization and in vitro differentiation of feline progenitor-like amniotic epithelial cell. *Stem Cell Res Ther* 4:133
- Sadeesh EM, Meena K, Fozia S, Yadav PS (2014a) 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
- Sadeesh EM, Meena K, Balhara S, Yadav PS (2014b) 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
- Saha A, Panda SK, Chauhan MS, Manik RS, Palta P, Singla SK (2012) Birth of cloned calves from vitrified-warmed zona-free buffalo (*Bubalus bubalis*) embryos produced by hand-made cloning. *Reprod Fertil Dev* 25:860–865
- Saikhun J, Kitiyanant N, Songtaveesin C, Pavasuthipaisit K, Kitiyanant Y (2004) Development of swamp buffalo (*Bubalus bubalis*) embryos after parthenogenetic activation and nuclear transfer using serum fed or starved fetal fibroblasts. *Reprod Nutr Dev* 44:65–78
- Seo MS, Park SB, Kim HS, Kang JG, Chae JS, Kang KS (2013) Isolation and characterization of equine amniotic membrane-derived mesenchymal stem cells. *J Vet Sci* 14:151–159
- 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
- Shambloot MJ, Axelman J, Wang S, Bugg EM, Littlefield JW, Donovan PJ, Blumenthal PD, Huggins GR, Gearhart JD (1998) Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc Natl Acad Sci USA* 95:13726–13731
- 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
- Srirattana K, Lorthongpanich C, Laowtammathron 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
- 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
- Takahashi M, Keicho K, Takahashi H, Ogawa H, Schultz RM, Okano A (2000) Effect of oxidative stress on development and DNA damage in in vitro cultured bovine embryos by comet assay. *Theriogenology* 54:137–145
- Tamagawa T, Oi S, Ishiwata I, Ishikawa H, Nakamura Y (2007) Differentiation of mesenchymal cells derived from human amniotic membranes into hepatocyte-like cells in vitro. *Hum Cell* 20:77–84

- Tamagawa T, Ishiwata I, Ishikawa H, Nakamura Y (2008) Induced in vitro differentiation of neural-like cells from human amnion-derived fibroblast-like cells. *Hum Cell* 21:38–45
- Tsuji H, Miyoshi S, Ikegami Y, Hida N, Asada H, Togashi I, Suzuki J, Satake M, Nakamizo H, Tanaka M, Mori T, Segawa K, Nishiyama N, Inoue J, Makino H, Miyado K, Ogawa S, Yoshimura Y, Umezawa A (2010) Xenografted human amniotic membrane-derived mesenchymal stem cells are immunologically tolerated and transdifferentiated into cardiomyocytes. *Circ Res* 106:1613–1623
- Vajta G (2007) Hand-made cloning: the future way of nuclear transfer. *Trends Biotechnol* 25:250–253
- Vassena R, Dee Schramm R, Latham KE (2005) Species-dependent expression patterns of DNA methyltransferase genes in mammalian oocytes and pre-implantation embryos. *Mol Reprod Dev* 72:430–443
- Violini S, Gorni C, Pisani LF, Ramelli P, Caniatti M, Mariani P (2012) Isolation and differentiation potential of an equine amnion-derived stromal cell line. *Cytotechnology* 64:1–7
- Wakayama T, Yanagimachi R (1999) Cloning of male mice from adult tailtip cells. *Nat Genet* 22:127–128
- 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 ntES cells derived from the same individuals. *J Reprod Dev* 51:765–772
- Wang M, Zhou Y, Tan WS (2010) Clonal isolation and characterization of mesenchymal stem cells from human amnion. *Biotechnol Bioprocess Eng* 15:1047–1058
- 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
- Yamahara K, Harada K, Ohshima M, Ishikane S, Ohnishi S, Tsuda H, Otani K, Taguchi A, Soma T, Ogawa H, Katsuragi S, Yoshimatsu J, Harada-Shiba M, Kangawa K, Ikeda T (2014) Comparison of angiogenic, cytoprotective, and immunosuppressive properties of human amnion- and chorion-derived mesenchymal stem cells. *PLoS ONE* 9:e88319
- 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
- Yang J, Yang S, Beaujean N, Niu Y, He X, Xie Y, Tang X, Wang L, Zhou Q, Ji W (2007) Epigenetic marks in cloned rhesus monkey embryos: comparison with counterparts produced in vitro. *Biol Reprod* 76:36–42
- Zhao XE, Zheng YM (2010) Development of cloned embryos from porcine neural stem cells and amniotic fluid-derived stem cells. *Animal* 4:921–929
- Zhou W, Xiang T, Walker S, Farrar V, Hwang E, Findeisen B, Sadeghieh S, Arenivas F, von Abruzeze RV, Polejaeva I (2008) Global gene expression analysis of bovine blastocysts produced by multiple methods. *Mol Reprod Dev* 75:744–758
- 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