

The effect of amniotic membrane stem cells as donor nucleus on gene expression in reconstructed bovine oocytes

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ABSTRACT Nuclear reprogramming of a differentiated cell in somatic cell nuclear transfer (SCNT) is a major concern in cloning procedures. Indeed, the nucleus of the donor cell often fails to express the genes which are a prerequisite for normal early embryo development. This study was aimed to evaluate the developmental competence and the expression pattern of some reprogramming related genes in bovine cloned embryos reconstructed with amniotic membrane stem cells (AMSCs) in comparison with those reconstructed with mesenchymal stem cells (MSCs) and adult fibroblasts (AF) as well as with *in vitro* fertilized (IVF) oocytes. *In vitro* matured abattoir-derived oocytes were considered as recipients and a hand-made cloning technique was employed for oocyte enucleation and nuclear transfer (NT) procedures. The expression pattern of genes involved in self-renewal and pluripotency (*POU5F1*, *SOX2*, *NANOG*), imprinting (*IGF2*, *IGF2R*), DNA methylation (*DNMT1*, *DNMT3A*), histone deacetylation (*HDAC2*), and apoptosis (*BAX*, *BCL2*) were evaluated in NT and IVF derived embryos. Despite the insignificant difference in cleavage rate between reconstructed and IVF oocytes, the blastocyst rate in the IVF group was higher than that of other groups. Among reconstructed oocytes, a higher blastocysts rate was observed in MSC-NT and AMSCs-NT derived embryos that were significantly higher than AF-NT derived ones. There were more similarities in the expression pattern of pluripotency and epigenetic modification genes between MSC-NT and IVF derived blastocysts compared with other groups. In conclusion, considering developmental competence, AMSCs, as alternative donors in SCNT procedure, like MSCs, were prone to have more advantage compared with AF.

KEY WORDS: nuclear transfer, reprogramming, bovine, imprinting, embryo

Introduction

Somatic cell nuclear transfer (SCNT) is an incompetent process in embryo production. There are numerous factors/causes affecting the success rate of SCNT procedure. Donor cells differentiation status is one of those factors that can influence the development of NT-derived embryos. It is thought that in nuclear transfer (NT), the nucleolus of undifferentiated stem cells may be more easily reprogrammed as the genome of reconstructed oocytes (Kumar *et al.*, 2007). Among many types of stem cells, mesenchymal stem cells (MSCs) are on the leading edge as they are easy to expand in culture while maintaining their stemness properties. Bovine MSC

as a first type of adult stem cells has been used in SCNT procedure with an appropriate nuclear reprogramming after NT and successful live birth. (Kato *et al.*, 2004). Cloned embryos derived from bovine and porcine undifferentiated MSCs have consistently resulted in high preimplantation development compared to adult fibroblasts (Colleoni *et al.*, 2005). Amniotic membrane stem cells (AMSCs), with their following biological characteristics such as low immu-

Abbreviations used in this paper: AF, adult fibroblast; AMSC, amniotic membrane stem cell; IVC, *in vitro* culture; IVF, *in vitro* fertilization; MSC, mesenchymal stem cell; SCNT, somatic cell nuclear transfer.

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Accepted: 6 April 2016.

nogenicity, low tumorigenicity upon transplantation, widespread availability, and lack of ethical problems associated with their use have attracted the attention of researchers (Toda *et al.*, 2007). Application of AMSCs in SCNT is limited to few studies on some species such as porcine (Zheng *et al.*, 2009). In these studies the characteristics of SCNT embryos in both types of nuclear donors, MSCs and AMSCs, have indicated their adequate reprogramming potential. However, it has already remained to be elucidated which of them is more appropriate in NT and how extent their differentiation status is crucial in cloning efficiency.

As known, the low efficiency of post implantation development of embryos following NT of differentiated cells, at least in part, is due to faulty nuclear reprogramming, leading to defects in early embryo gene expression (Rodriguez-Osorio *et al.*, 2012, Samiec and Skrzyszowska, 2005). During normal development, early embryos undergo a well-orchestrated series of DNA methylation and histone modification that are believed to play an important role in establishing a condition in which the chromatin will be permissive to early embryonic gene expression. In contrast, NT-derived embryos typically show abnormal patterns of DNA methylation and histone modifications and the consequent abnormal expression of genes in early embryo (Bortvin *et al.*, 2003, Jaenisch *et al.*, 2004). Studies on pre-implantation development of SCNT embryos have shown significant deficiencies in many parts of nuclear events, including transcription and translation, epigenetic modifications such as methylation of DNA, acetylation of histones and chromatin configuration as well as genetic imprinting (Kumar *et al.*, 2007). In support of these observations, mammalian NT-derived embryos displayed so many abnormalities caused by aberrant expression of genes involved in early development and epigenetic reprogramming (Niemann *et al.*, 2008). However, the amount of data collected associated with gene expression in preimplantation cloned embryos, reconstructed with AMSCs, is limited to a few genes. Accordingly, in the present study, we aimed to evaluate and compare the developmental competence as well as the expression of panel of developmentally important genes involved in early embryo development in resulting blastocysts derived from bovine oocytes reconstructed with amniotic membrane stem cells (AMSCs), mesenchymal stem cells (MSCs), and adult fibroblasts (AF).

Results

The stemness status of AMSCs and MSCs was confirmed after their differentiation to adipocytes and osteocytes followed by Oil red and Alizarin red staining, respectively. Their stemness status was also confirmed by the presence of OCT4 protein by immunocytochemistry method.

As shown (Table 1), after 24h of culture there was a significant difference in cleavage rate between groups ($p < 0.05$); so that among NT embryos those derived from AMSCs and AF had the highest and lowest cleavage rate, respectively. The difference among groups, however, was lost in the next day.

Despite the lower developmental of NT embryos compared to IVF ones ($p < 0.05$), the use of stem cells (MSCs or AMSCs) improved developmental competence of reconstructed embryos compared to fibroblast ($p < 0.05$). However, there was no significant difference between AMSCs-NT and MSCs-NT embryos. As shown (Table 1), the lowest blastocyst rate was also observed in the AF-NT group compared to other groups.

TABLE 1

DEVELOPMENTAL RATE IN BOVINE IVF AND NT EMBRYOS

Groups	Oocyte No.	Cleavage rate (%)		Blastocyst rate (%)	
		Day 1	Day 2	Day 7	Day 8
IVF	438	NA	74.34±1.36	23.6±0.71 ^a	26.72±0.64 ^a
AF-NT	363	42.51±2.79 ^a	73.7±1.19	9.27±0.89 ^b	12.65±0.64 ^b
MSC-NT	370	48.72±1.85 ^b	79.79±2.36	16.48±2.09 ^c	19.23±1.65 ^c
AMSC-NT	328	56.40±1.26 ^c	80±3.56	16.1±2.99 ^c	17.95±3.01 ^{bc}

^{A,B,C} Different superscripts in the same column denote a significant difference ($p < 0.05$). Data represent n=6 replicates.

The relative abundance (RA) of transcripts at the blastocyst stage in cloned and IVF embryos has been shown in Fig. 1A-J.

As shown, the expression of *POU5F1* in IVF derived blastocysts was higher than NT derived ones. Though, the difference was in significant between IVF and MSC-NT derived embryos.

The highest and lowest expression of *NANOG* were observed in AMSC-NT and IVF derived embryos, respectively ($p < 0.05$). No significant difference was observed between AF-NT and MSCs-NT derived embryos.

Similarly, the highest and lowest expression of *SOX2* were observed in AMSC-NT and IVF derived embryos, respectively ($p < 0.05$). However, there was no significant difference between NT derived blastocysts.

The relative abundance of *DNMT1* transcript was significantly ($p < 0.05$) higher in AF-NT and AMSC-NT than IVF and MSC-NT embryos. There was no significant difference between AF and AMSC-NT and between IVF and MSC-NT embryos.

The RA of *DNMT3A* was similarly higher in AF-NT and AMSCs-NT embryos compared to IVF and MSCs-NT groups. However, the difference between MSCs-NT and AMSCs-NT embryos was insignificant.

HDAC2 exhibited high level of expression in IVF embryos compared to NT derived counterparts, ($p < 0.05$) and no differences were found between NT groups.

The RA of *IGF2* transcript was similar between groups and no significant differences were observed. The expression of *IGF2R* in AF-NT embryos was significantly higher than other groups.

No significant difference in *BAX* expression was observed between groups, while the expression of *BCL2* in AF-NT derived blastocysts was significantly lower than other groups.

Discussion

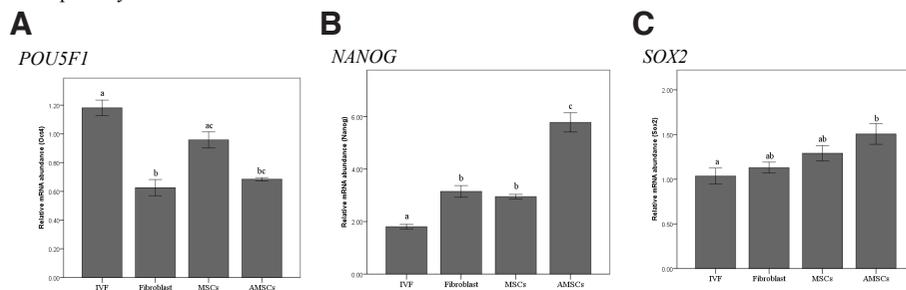
Somatic cell nuclear transfer (SCNT) efficiency in livestock is relatively low. In cattle only 1.7% of reconstructed oocytes and 11.5% of transferred NT embryos develop to term, and some of them exhibiting abnormalities at birth (Keefer, 2015). The main obstacle in SCNT procedure is thought to be in appropriate reprogramming of the donor nucleus (Niemann *et al.*, 2008). Among different component of SCNT, type of donor cell and its differentiation status may influence the quality of resulting embryo and its subsequent postimplantation development. While, some studies have shown that there is no difference between fully differentiated and less differentiated donor cells in SCNT procedure, there is evidence indicating a less differentiated donor cell can increase SCNT efficiencies compared with terminally differentiated cell types.

In the present study application of fibroblast as donor cell in NT procedure using HMC method resulted in 12.65% blastocyst. Despite lack of study using this method in production of cattle NT embryos, the application of this method using AF-NT reconstructed oocytes resulted in 8.8% to 13.2% and 19.5% blastocyst rate in sheep (Hosseini *et al.*, 2013a, Hosseini *et al.*, 2013b) and goat (Hosseini *et al.*, 2015), respectively. Regarding the use of stem cells as donor cells, the porcine MSC-NT reconstructed oocytes had a higher developmental rate compared to the fetal fibroblast-NT derived counterparts (Jin *et al.*, 2007, Samiec *et al.*, 2015). The higher appropriate remodeling of stem cell-NT derived embryos has proved in different species (Bosch *et al.*, 2006, Faast *et al.*, 2006, Kumar *et al.*, 2007, Kumar *et al.*, 2012, Lee *et al.*, 2006, Rideout

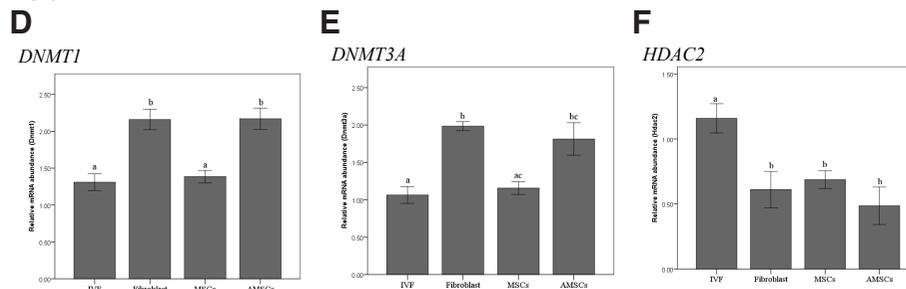
et al., 2000). Some reports, however, have rejected transcendence of stem cells over somatic cells in normal reprogramming of NT derived embryos (Berg *et al.*, 2007, Li *et al.*, 2013, Sung *et al.*, 2006). In the first report on the use of bovine MSCs for NT, no priority in developmental potential was observed in stem cell derived embryos compared to non-stem cell derived counterparts (Kato *et al.*, 2004). On the contrary, cloned embryos derived from bovine and porcine undifferentiated MSCs and their derivatives gave rise to the higher preimplantation development compared to adult fibroblasts (Colleoni *et al.*, 2005).

In our study condition, despite the lower developmental rate of NT derived embryos compared to the IVF derived ones, the use of stem cells (MSCs or AMSCs) as donor cells could improve developmental competence of reconstructed oocytes compared to those reconstructed with fibroblasts.

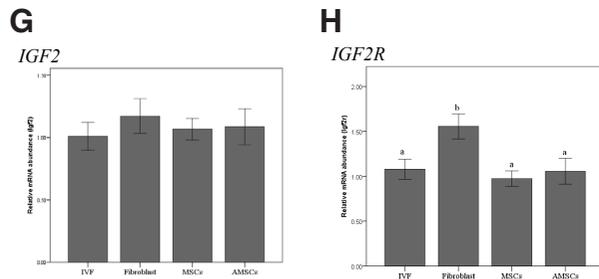
Pluripotency



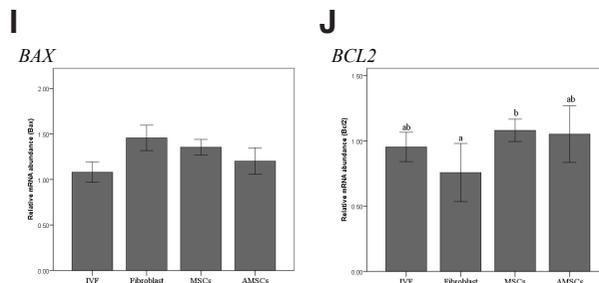
Epigenetic modifications



Imprinting



Apoptosis



To the best of our knowledge, this study is the first report on the use of AMSCs as a donor in bovine NT that compare mRNA expression of a panel of developmental-related genes among cloned blastocysts derived from different donor cells. However, further data on post implantation development are needed to shed more light on the effect of the stem cell nature of the donor nucleus in SCNT. In the only report on the use of amnion derived stem cells for NT, the resulting porcine blastocyst and pregnancy rates were higher than those reconstructed with fully differentiated somatic cells (Zheng *et al.*, 2009). Aberrant gene expression is one of the suggested causes of the low success rates of development in NT embryos of mammals.

During normal development, the early embryo undergoes a series of coordinated epigenetic modifications (e.g. DNA methylation and histone modifications), which plays an important role in creating an appropriate chromatin status for gene expression in early embryo. Considering the occurrence of some abnormalities in gene expression of SCNT embryos, in the present study the expression of some genes involved in reprogramming, epigenetic modifications, apoptosis, and imprinting were assessed as indicators of quality of NT produced embryos.

POU5F1 or *OCT4* (octamer-binding transcription factor 4) as a marker for undifferentiated cells, critically involved in self-renewal and pluripotency of stem cells across mammalian

Fig. 1. Relative abundance of *POU5F1* (A), *NANOG* (B), *SOX2* (C), *DNMT1* (D), *DNMT3A* (E), *HDAC2* (F), *IGF2* (G), *IGF2R* (H), *BAX* (I) and *BCL2* (J) transcripts in bovine blastocyst derived from IVF and NT (AF, MSCs, and AMSCs). mRNA from pools of 4 embryos at the blastocyst stage were reverse transcribed, and subjected to real-time quantitative PCR. All reactions were normalized for histone H2A mRNA expression. Values with superscripts "abc" refers to significant ($p < 0.05$) differences in relative transcript abundance between groups.

species and is presumed to be a critical factor controlling murine, porcine, and bovine preimplantation embryonic development. It has been shown that the *POU5F1* expression in cloned embryos at blastocyst stage is lower than *in vivo* and IVF derived blastocysts. In porcine, *POU5F1* expression in MSCs-NT and AF-NT derived blastocysts was significantly decreased compared with IVF and *in vivo* derived counterparts (Kumar et al., 2007); Whereas, the low expression of *POU5F1* is associated with low developmental competence of embryos, a decreased *POU5F1* expression in NT derived embryos might be the reason for their lower blastocyst rates compared to the IVF derived embryos. Since the methylation status of gene promoter is associated with its expression, the higher expression of *POU5F1* in IVF and MSC-NT derived blastocysts might be related to the lower expression of *DNMT1&2* in these groups. It has been shown that porcine bone marrow MSCs with high level of *POU5F1* expression are more potent as NT donor cells and that *POU5F1* transfection of MSCs has improved the developmental competence of NT derived embryos (Lee et al., 2014). It has also been shown that *POU5F1* expression in bovine fibroblasts positively correlates with blastocysts formation and total cell number of NT derived embryos (Rodriguez-Alvarez et al., 2013). Therefore, the higher blastocysts rates in IVF and MSCs-NT derived embryos might be related, at least partly, to the higher expression of *POU5F1* in these groups.

SOX2 as another gene involved in pluripotency, is a transcription factor that is essential for maintaining pluripotency and self-renewal of stem cells and also plays a key role in many stages of mammalian development. In the present study, despite no significant difference in *SOX2* expression between IVF and AF and MSC-NT embryos, the expression in AMSCs-NT derived blastocyst was higher than IVF embryos. It seems the expression of this gene is not associated with survival rate (Kumar et al., 2012) so that the lower *SOX2* expression in IVF group has no adverse effect on embryo development in this group. This finding was consistent with previous report indicating the low expression of *SOX2* in porcine IVF, parthenogenetic, and NT embryos reconstructed with MSCs or fetal fibroblast (Kumar et al., 2012). However, the higher expression of this gene in post-hatching stages (Kumar et al., 2012) may indicate its more important role during or after implantation.

NANOG is a homeobox-containing transcription factor with an essential function in maintaining the pluripotent status in inner cell mass as well as ESCs (Mitsui et al., 2003). It has been shown that overexpression of *NANOG* in donor cells had no effect on blastocyst rate in reconstructed embryos (Zhang et al., 2011). In our present study, similarly, the higher expression of *NANOG* in cloned AMSC-NT embryos could not improve the embryo development. There is evidence indicating the culture system may affect the *NANOG* expression. For instance its expression might be affected by differences in IVF and NT culture systems (group culture in IVF and WOW in NT system). This hypothesis that how extent the concentration of metabolites such as ammonium may be influenced by the culture system and how it may influence the expression of *NANOG* need to be further investigated.

As shown in contrast to *SOX2* and *NANOG*, the expression of *POU5F1* in IVF derived blastocyst was higher than SCNT derived counterparts, except for MSC-NT embryos and this might be the reason for the higher blastocyst rate in MSC-NT group compared to other SCNT groups.

Among DNA-methyltransferases, *DNMT1* as abundant DNA

methyltransferase is responsible for copying methylation patterns following DNA synthesis, whereas *DNMT3A&B* are involved in de novo methylation (Dean et al., 1998). Previous studies have shown a correlation between incomplete DNA methylation and the lack of NT success in mammals (Bortvin et al., 2003, Dean et al., 2001). *DNMT1* and *DNMT3A* mRNA were continuously identified in *in vitro* produced bovine embryos from 2-cell to the blastocyst stages (Golding and Westhusin, 2003). The relative abundance of the *DNMT1* transcript, however, was significantly lower in *in vivo* derived bovine embryos compared with *in vitro* counterparts (Wrenzycki et al., 2001a). In our study, the lower expression of both *DNMT1* and *DNMT3A* in IVF and MSCs-NT derived blastocysts compared to AF-NT and AMSC-NT counterparts may be related to the higher developmental competence of the blastocysts in the former groups due to the higher chance of expression of developmentally important genes. In some previous reports there are evidences indicating similarities in *DNMT1* expression between *in vivo* and *in vitro* embryos and that the amount of *DNMT3A* transcripts in bovine IVF embryos is higher than NT embryos (Rodriguez-Osorio et al., 2009). In porcine, SCNT derived blastocyst using terminally differentiated somatic cells had a higher *DNMT1* expression compared to the either IVF or stem cell-NT derived blastocysts as well as *in vivo* produced embryos (Zhu et al., 2004). In agreement with our study, *DNMT3A* expression in porcine IVF embryos was not significantly different with MSC-NT counterparts (Lee et al., 2014).

From above is not easy to draw a final conclusion about the consequences of lower expression of DNMTs in IVF and MSCs-NT derived blastocysts on further post hatching embryo development. One possibility for the lower *DNMT1&3A* in MSCs-NT derived blastocysts compared to AF and AMSCs-NT derived counterparts might be related to the difference in methylation status of donor cells in these groups. It seems the methylation status of donor cells tend to be remained in SCNT embryos even after several cell divisions in embryos. In support of this, in blastocysts derived from treated MSCs in which the *DNMT1* expression was downregulated, *DNMT1* expression was lower than cloned blastocysts derived from untreated MSCs (Kim et al., 2014). Therefore, in cattle cloned embryos there is a tendency to preserve the DNA methylation patterns inherited from their donor cells (Bourc'his et al., 2001).

Histone acetylation is regulated by two classes of enzymes, the histone acetyltransferases (HAT's) and histone deacetylases (HDAC's). Histone Acetylation facilitates chromatin decondensation so that makes the DNA more accessible to transcriptional factors, while HDAC's through histones deacetylation counteract this effect (Wolffe and Pruss, 1996). In the present study, in contrast to DNMTs, *HDAC2* exhibited a high level of expression in IVF embryos compared to NT counterparts and no differences were found between NT groups. In agreement to this finding, in pig and cattle the *HDAC2* expression in IVF embryos was higher than cloned embryos (Kumar et al., 2007). The abnormal reprogramming due to aberrant histone modifications following SCNT has been documented by other studies (Enright et al., 2005, Santos et al., 2003).

The *IGF2* and *IGF2R* genes as maternally and paternally inherited genes, respectively, are among the best studied imprinted genes involved in fetal growth regulation, and are essential for normal development (Latham et al., 1994). *IGF2* is a classic imprinted gene as it appears to be biallelically transcribed up to the morula stage, but in the blastocyst stage the maternal *IGF2* allele is silenced. Aberrant expression of imprinted genes may be responsible for devi-

ated growth characteristics seen in fetuses and offspring originated from embryos produced in *in vitro* systems, including SCNT derived embryos (Niemann *et al.*, 2002). In our study, the *IGF2* expression was similar in all studied groups, while the expression of *IGF2R* in AF-NT blastocysts was greater than other groups. In porcine, despite no difference in *IGF2R* expression in IVF, parthenogenetic, and SCNT derived blastocysts, the expression in *in vivo* derived blastocysts was higher than *in vitro* derived counterparts (McElroy *et al.*, 2008). In one report no significant difference was observed in *IGF2R* expression between bovine IVF and cloned embryos (Niemann *et al.*, 2002). There are also evidences indicating the higher *IGF2R* expression, in *in vitro*-produced bovine and porcine embryos (Bertolini *et al.*, 2002, Kumar *et al.*, 2007), with no differences in expression levels between IVF and SCNT blastocysts (Han *et al.*, 2003, Kumar *et al.*, 2007, Wrenzycki *et al.*, 2001b). It was demonstrated that any *in vitro* manipulation of the embryo might induce epigenetic modifications in various developmentally important genes including imprinted genes (Feil, 2001, Niemann *et al.*, 2002). There are also evidences indicating the effect of culture systems on expression pattern of imprinted genes. For instance, extended culture of murine embryos in deficient medium (e.g., Whittens' medium) led to biallelic expression of the H19 and that the vicinity of embryos in group culture increased ammonium concentration which in turn could induce aberrant expression of the imprinted genes in blastocysts (Doherty *et al.*, 2000).

Another group of genes which play an important role during embryo development were pro-apoptotic (*BAX*) and Anti-apoptotic (*BCL2*) genes (Metcalf *et al.*, 2004). In our study the *BAX* expression in IVF derived blastocysts was similar to NT embryos, while the transcript abundance of *BCL2* in AF-NT blastocysts was significantly lower than MSC-NT embryos whereas no differences were detected between IVF, MSC-NT, and AMSC-NT embryos.

In porcine, the *BAX* expression was significantly higher in FF-NT embryos than in MSC-NT and IVF derived embryos (Kumar *et al.*, 2007). There are also evidences indicating the relationship between total cell number, embryo fragmentation, number of apoptotic cells, and developmental potential of embryos with expression of cell death regulatory genes, *BAX* and *BCL2* (Jin *et al.*, 2007). The elevation and reduction in expression of *BAX* and *BCL2*, respectively, is more evident in cloned embryos than their IVF counterparts (Kumar *et al.*, 2007). Considering the lack of difference in *BAX* expression between groups, the lower developmental competence in AF-NT derived embryos might be related to the lower expression of *BCL2* in this group.

As discussed above, in our study condition the expression of the majority of genes in IVF and NT derived blastocysts were different. Indeed, the most similarities in gene expression between NT and IVF groups were seen in MSC-NT blastocysts, especially the expression of *POU5F1*, *DNMT1*, and *DNMT3A*. In the other hand, the higher developmental competence of oocytes reconstructed with MSCs compared to other NT oocytes, might be related, at least partly, to the similarities in genes expression between MSCs-NT and IVF groups. Additionally, application of AMSCs as donor cell in SCNT procedure was more appropriate compared with adult fibroblast.

Materials and Methods

Chemicals and media

Except where otherwise indicated, all chemicals were obtained from the Sigma (St. Louis, MO, USA).

Experimental design

The abattoir derived oocytes after IVM were subjected either to IVF/IVC, as control, or enucleated as recipient cytoplasts for 3 different donor cells. For NT, all donor cells were used in the third passage. The IVF and chemically activated reconstructed oocytes were separately cultured for 8 to 9 days. The cleavage and blastocyst rates were recorded on days 3 and 7 to 8 after IVC, respectively. The resulting blastocysts were then subjected to RNA extraction and quantification using RT-PCR method.

Donor cell preparation

Adult fibroblast cells

Preparation of AF was performed according to Heidari *et al.*, method (2010). Briefly, ear skin biopsy samples of a 12-month-old bull was washed in Ca^{2+} and Mg^{2+} free PBS and finely minced with a sterile scalpel blade. The explants transferred to 60x10 mm Falcon TM plastic Petri dishes (Falcon 3004; Becton Dickinson) and cultured in DMEM containing 40% fetal calf serum (FCS; Gibco 10270) supplemented with 100IU/ml penicillin and 100 μ g/ml streptomycin, 2mM L-glutamine (Gibco, Grand Island, NY), and 25mM $NaHCO_3$ at 38.5°C and 5% CO_2 . After 48h, the culture medium was replaced by DMEM medium supplemented with 10% FCS. Fibroblast cells began to outgrow from the explants by the third day in culture and the explants were removed from the Petri dishes on day 6. After explants removal, the cells were harvested at 60% confluency using trypsin/EDTA in PBS, counted and seeded at 5×10^5 cells into 25cm² tissue culture flasks. The first passage was carried out at 90% confluency and 1×10^6 cells/cryovial was considered for cryopreservation in DMEM containing 40% FCS and 10% DMSO.

Amniotic membrane stem cells

The AMSCs were obtained from amniotic membrane at 75-90 days of pregnancy. The amniotic membrane was separated under a stereomicroscope into epithelial and connective tissues. AMSCs were obtained from epithelial tissue following 30 minutes of enzymatic digestion in DMEM containing collagenase type IV (1mg/ml), hyaluronidase (0.5mg/ml), trypsin (1mg/ml), and DNase (50IU/ml). After 10 minutes centrifugation at 500xg, isolated cells were resuspended in DMEM containing 20% FCS and 5ng/ml fibroblast growth factor (FGF) and were cultured at 38.5°C in 5% CO_2 . After 7 days of culture, nonadherent cells were removed and the medium was refreshed every 2 days up to 80-90% confluency. At desired confluency, the cells were isolated by incubation in 0.25% Trypsin/EDTA for 10 minutes and were then plated at 2×10^4 cells/ml.

The stemness status of AMSCs was confirmed by Oct-4 expression, using specific primary antibody, monoclonal anti-Oct-4 (Abcam, ab18976), and FITC-conjugated secondary antibody. The cell nuclei were also stained with 5 μ g/ml H33342 and finally, the stained cells were analyzed under fluorescent microscope. The stemness status of AMSCs at third passage was also confirmed by their osteogenic and adipogenic differentiation. Osteogenic medium was comprised of 10% FCS, 150 μ g/ml β -glycerophosphate, 50 μ g/ml ascorbic acid, and 10^{-8} M dexamethasone, and adipogenic differentiation medium was consisted of 2% FCS and 100IU/ml ITS. Osteogenic and adipogenic differentiation were assessed by colorimetric visualization of calcium sediment (Alizarin Red S stain) and intracellular accumulated lipid-rich vacuoles (Oil red) in culture plates, respectively.

Mesenchymal stem cells

The adipose tissue from lumbar paravertebral regions after washing 3 times in PBS was minced into small pieces and subjected to enzymatic digestion using collagenase type IV (1mg/ml), hyaluronidase (0.5mg/ml), trypsin (1mg/ml), and DNase (50IU/ml) at 37°C for 30min. The digested specimen was centrifuged at 500g for 5min and the cellular pellet after washing, three times, were cultured in DMEM medium at 38.5°C and 5% CO_2 . After 3 days of culture, nonadherent cells were removed and the medium was refreshed every 3 days until the culture became confluent. Assessment of stemness status of MSCs was similar to AMSCs.

TABLE 2

**SEQUENCE AND ANNEALING TEMPERATURE
OF PRIMERS USED FOR RT-PCR**

Gene	Gen Bank accession no.	Primer sequence (5'-3')	Product size (bp)	Annealing temp. (°C)
<i>Histone H2A</i>	XM_002686056.3	F-CCAATATCCAGGCCGTGTTG R-GGAGAGCTGGGTAGGACTTG	97	60
<i>POU5F1 (Oct 4)</i>	NM_174580.2	F-TGGACAAGGAGAAGCTGGAG R-ACATCGGCCTGGGTATATCC	130	59
<i>NANOG</i>	NM_001025344.1	F-CATCTGCTGACACCCCTCGAC R-CACTCTGGGGCTTGTGGAAG	101	62.5
<i>SOX2</i>	NM_001105463.2	F-GACCTACATGAACGGCTCGC R-AGCTGGCCTCGGACTTCA	107	60
<i>DNMT1</i>	NM_182651.2	F-GTGTGAGCCGAGTGAACCTGG R-GATGGCCAAAGTGCTCCCG	130	62.5
<i>DNMT3A</i>	NM_001206502.1	F-TCCGGGTGCTGTCTCTATTC R-TGATGGAGTCTCACACACC	111	62.5
<i>HDAC2</i>	NM_001075146.1	F-GAGGCGGCAAGAAGAAAGTC R-GTTATGGGTCATGCGGATCC	107	60
<i>IGF2</i>	NM_174087.3	F-GTGACCTGTGGCCCTTGATC R-CACCGACTTCTCTGCTGTGA	96	62.5
<i>IGF2R</i>	NM_174352.2	F-CCGTATCACCGAGTACCTG R-CGAAGATGACCGAGGCGTAG	126	59
<i>BAX</i>	NM_173894.1	F-GTGCCCGAGTTGATCAGGAC R-AAGTAGGAGAGGAGGCCGT	110	59
<i>BCL2</i>	NM_001166486.1	F-CTTCGCCGAGATGCCAGTC R-TCACCCCGTCCCTGAAGA	95	59

Oocyte in vitro maturation

In vitro maturation (IVM) of bovine oocytes was carried out as described previously (Shirazi et al., 2009). In brief, all visible follicles with a diameter of 2–9 mm of abattoir-derived ovaries were aspirated using a 10 ml syringe fitted with an 18 gauge needle. The aspiration medium was preincubated HEPES-buffered tissue culture medium-199 (HTCM-199) supplemented with penicillin/streptomycin and 50IU/ml heparin. The cumulus–oocyte complexes (COCs) with at least three layers of cumulus cells engulfing oocyte with evenly granulated cytoplasm were selected for the experiments. Ten to 15 selected COCs were *in vitro* matured in 50µl of TCM199 supplemented with 10% FCS and 0.1IU/ml FSH in 5% CO₂ atmosphere at 39°C.

Oocyte enucleation

After 20–22h IVM, oocytes were denuded by vortexing in HTCM-199 supplemented with 10% FCS and 0.3mg/ml hyaluronidase. The oocytes with visible degenerative changes or physical damage were discarded from the experiments. Zona pellucida was removed, by incubation of oocytes for 2–3min in medium containing 0.25% pronase.

For induction of metaphase plate protrusion, bovine zona-free oocytes were incubated in enucleation medium containing 0.5µg/ml demecolcine for 90min. The oocyte enucleation was performed by removing the protruded cytoplasm under a stereomicroscope using finely drawn hand-made pipettes as described by Hosseini et al., (Hosseini et al., 2013b). In brief, zona-free oocytes with cytoplasmic protrusion were placed in groups of ten in enucleation medium under mineral oil. First the oocyte was moved by the tip of the pipette in such a way that the cytoplasmic protrusion was placed adjacent to the enucleation pipette. The pipette tip was then attached to the cytoplasmic protrusion so that the protruded cytoplasm is drawn into the needle by gentle mouth suction. The enucleation was then simply accomplished by removing the pipette tip from the enucleation droplet to the mineral oil. The enucleation was then checked after incubation of remaining cytoplasm in 5µg/ml H33342 for 5min under an epifluorescent microscope.

Cell attachment

The serum starved donor cells at the third passage were considered for NT. Fifty to one hundred donor cells were added to a drop of HTCM-199 containing 10mg/ml Phytohemagglutinin (PHA-P). The cytoplasts (enucleated oocytes) were individually rolled over a single donor cell to produce donor cell–recipient cytoplasm couplets. The couplets were then transferred into wash drops (HTCM-199 supplemented with 10% FCS, 30µg/ml PVA, and 50µg/ml BSA) before being incubated in fusion buffer (0.3M mannitol, 100µM MgSO₄, 50µM CaCl₂, 500µM HEPES, 0.05% BSA) for 1min. The 10–15 couplets were then placed in a fusion chamber (electrodes 0.5mm apart) and after being manually aligned were received AC pulse (7V for 2sec). Fusion was induced by applying two DC electrical pulses (1.5KV/cm for 30µsec; BLS). After electrofusion, the couplets were incubated in HTCM-199 supplemented with 20% FCS for 30min and then processed for activation and embryo culture.

Artificial activation and culture

Thirty minutes after fusion, reconstructed oocytes were transferred to HSOF medium containing 5µM ionomycin for 4 min. They were then washed in HSOF containing 30mg/ml BSA to inactivate ionomycin and then incubated in 2mM 6-dimethylaminopurine prepared in SOFaaBSA (SOF supplemented with 2mM glutamine, essential and non-essential amino acids and 8mg/ml BSA) medium for 4h. Following activation, five to six reconstructed embryos were washed and separately cultured based on well of the well (Vajta et al., 2000) culture system in 20µl droplets of SOFaaBSA medium under oil at 39°C in an atmosphere of 5% CO₂, 7% O₂, and 88% N₂. On Day 3, the culture medium was refreshed with the medium containing 5% charcoal-stripped FCS. The cleavage rate was recorded 24 to 48 h post activation and development to the blastocyst stage was assessed on days 7 and 8. Day 7 blastocysts were considered for subsequent molecular evaluation.

In vitro embryo production

Bovine IVF derived embryos was produced as described previously (Shirazi et al., 2009). In brief, the *in vitro* matured oocytes were exposed to motile spermatozoa obtained by centrifugation of frozen–thawed semen on a discontinuous Percoll density gradient (40% and 90%) at 700g for 20 min. During IVF, oocytes were cultured in TALP medium supplemented with 6mg/ml BSA and incubated with motile spermatozoa at 1×10⁶ spermatozoa/ml concentration for 22–24h at 39°C in 5% CO₂. After fertilization, presumptive zygotes were mechanically denuded and cultured in SOFaaBSA medium in 5% CO₂, 7% O₂, and 88% N₂ for 9 days.

Molecular evaluation**RNA extraction and cDNA synthesis**

Total RNA was extracted from pools (six replicates) of 4 seven-day-old embryos at expanded blastocyst stage derived from NT and IVF, using RNX Plus reagent (RN7713C; Sinaclon Bioscience, Karaj, Iran). Briefly, samples were lysed in 300µl of this reagent and mixed with 70µl chloroform. The resulting mixture was centrifuged (12000rpm, 4°C, 15 min), yielding an upper aqueous phase containing total RNA. After addition of 120µl isopropanol and 4µg/µl glycogen to supernatant, it was centrifuged (12000rpm, 4°C, 15 min) and the RNA pellet was washed with 75% ethanol. The RNA samples were re-suspended in 20µl DEPC-treated water and treated with RNase-free DNase (Sinaclon Bioscience, Karaj, Iran) to avoid amplification of contaminating genomic DNA. The amount and quality of RNA were determined by spectrophotometry (Amersham Pharmacia Ultrospec 1100 Pro). Only RNA of sufficient purity, having an absorbance ratio (A₂₆₀/A₂₈₀) greater than 1.9, was considered for synthesis of cDNA. Total RNA was reverse transcribed into cDNA in a short time after extraction (less than 2h) using M-MLV reverse transcriptase (Sinaclon Bioscience, Karaj, Iran) as described by Hassanpour et al., (2015) (Hassanpour et al., 2015) (Hassanpour et al., 2015). The reverse-transcription was done in a 20µl volume containing 10µl (14µg) of extracted RNA and 1µl random hexamer. This mixture was heated to 70°C for 5 min, and then 0.5µl of RNase inhibitor, 2µl RT buffer (50mM Tris-HCl, 75mM KCl, 3mM MgCl₂), 2µl dNTP (10mM) and 1µl M-MLV reverse transcriptase were added. This mixture was incubated for 5min at 25°C, followed by 60min at 42°C. The mixture was heated to 70°C for 10 min to denature the RNA and then stored at -20°C.

Quantitative real-time PCR

Real-time PCR was performed in two replicates for each sample (Rotor Gene Q 6000, Qiagen, USA). Primer sequences, the GenBank accession numbers, the size of amplified products, and annealing temperature of each primer are shown in Table 2. Half μ l DNase I treated cDNA (containing 0.35 μ g) was added to 10 μ l of SYBR Premix Ex Taq II Mix and 0.75 μ M of each specific primer in a total volume of 20 μ l. The PCR program was comprised of 40 cycles of 94°C for 40s, 59-62.5°C for 30s (annealing temperature; table 1) and 72°C for 30s.

Considering the selection of an appropriate housekeeping gene as a reference gene for normalization, there are several studies demonstrating that Histone H2a gene is highly reliable for analysis of relative gene expression in bovine embryos at blastocyst stage [Gutierrez-Adan *et al.*, 2004; Rizos *et al.*, 2002] and also for comparing the gene expression between bovine cloned and IVF embryos [Ross, *et al.*, 2010]. It is of note that the level of H2A mRNA is itself an indicator of the proportion of DNA-replicating cells in the analysed embryos. Melt curve analysis was conducted to confirm the specificity of each product. The no-template control and no-reverse transcriptase control were considered to check contamination of the PCR reagents. Data were analyzed using LinReg PCR software version 2012.0 (USA), to give the threshold cycle number (Ct). Mean efficiency values (E) for each gene were also determined from the amplification profiles of individual samples using the same software (Ruijter *et al.*, 2009). The following formula was applied to determine the relative gene expression in cloned embryos compared to the control group (IVF embryos) (Dorak, 2007, Pfaffl, 2001).

$$\text{Ratio} = \frac{E_{H2A}(C_t \text{ cloned embryos})/E_{\text{target}}(C_t \text{ cloned embryos})}{E_{H2A}(C_t \text{ IVF embryos})/E_{\text{target}}(C_t \text{ IVF embryos})}$$

Statistical analysis

The differences in relative abundance of gene expression and the percentages of embryonic development between groups were analyzed using one-way analysis of variance (ANOVA) after ArcSin transformation with SPSS software version 20.0.0 (IBM Corp.; USA). Data were expressed as mean \pm SEM. Differences were considered significant at $p < 0.05$.

Acknowledgments

The authors thank the Research Institute of Animal Embryo Technology for technical and financial supports, Shahrekord University.

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