Relative expression of cell growth regulatory genes insulin-like growth factors (IGF-1 and IGF-2) and their receptors (IGF-1R and IGF-2R) in somatic cell nuclear transferred (SCNT) and in vitro fertilized (IVF) pre-implantation buffalo embryos

Alok Pandey, Namita Singh, S.C. Gupta, J.S. Rana, Neelam Gupta

Abstract

Relative mRNA transcript expression of insulin-like growth factors, IGF-1, IGF-2 and their receptors, IGF-1R and IGF-2R, was studied in SCNT and IVF buffalo embryos at different developmental stages using SYBR green with real-time PCR. SCNT embryos were produced by enucleating IVM oocytes and transferring granulosa cells (passage 5) followed by the electrofusion and chemical activation method. IVF embryos were produced by culturing 15–20 COCs in BO capacitated sperms from frozen and thawed buffalo semen. SCNT embryo production rate was lower than IVF. IGF-1 mRNA expression was significantly upregulated at 2-cell, 16-cell, morula and blastocyst stages of SCNT embryos than in IVF embryos. IGF-1R expression declined from the 2-cell to the 16-cell stage, an increase and was highest in IVF blastocysts. Similar regulation was observed at different stages in SCNT embryos, except at the 4-cell stage where expression was higher. IGF-2 expression decreased up to the 8-cell stage and increased until the blastocyst stage, being higher in SCNT than IVF embryos. IGF-2R mRNA transcript expression was consistently lower in SCNT than in IVF embryos. Reprogramming of IGF-1, IGF-1R, IGF-2 and IGF-2R expression may have a significant role in cell proliferation in cloned embryos and is developmentally regulated.

Keywords: SCNT; IVF; IGFs; Buffalo; Epigenetic reprogramming

1. Introduction

Cloning by use of adult somatic cell nuclei (Wilmut et al., 1997) was a landmark discovery, but the technique still has limited applications because of lower viability of embryos and aberrations in neonates. Clones have now been produced using different donor cells in sheep (Wilmut et al., 1997; Schnieke et al., 1997), cattle (Cibelli et al., 1998; Kato et al., 1998; Wells et al., 1998; Kubota et al., 2000), goat (Baguisi et al., 1999), mice (Wakayama et al., 1998), pigs (Polejaeva et al., 2000; Onishi et al., 2000), and gaurs (Lanza et al., 2001), yet only <3% of cloned embryos result into live births (McElroy et al., 2008). This has been attributed to loss in ability of the transplanted nuclei from donor cell to enucleated oocyte in SCNT for normal embryonic development as the zygotic cleavages progress.

Pre-implantation embryonic development is initially under the control of genetic information stored in the ooplasm during oogenesis (Wrenzycki et al., 2005). Genetic programme of development at the 2- to 4-cell stage becomes dependent on new transcripts derived from activation of embryonic genome (Hoffmann et al., 2006). In the case of IVF or SCNT embryos, the early steps in development, including the timing of first division, are regulated by maternal gene expression that is essential for normal development.
cleavage, activation of the embryonic genome, compaction and blastocyst formation, may be affected by culture media and conditions, as well as the production procedure itself (Lonergan et al., 2003).

The fundamental problem in the transformation of highly differentiated somatic cell nuclei into totipotent blastomeres during early embryonic stages in comparison to the normal fertilized zygotes is the aberrant reprogramming process (Amaranth et al., 2007). Abnormalities in gene expression patterns in cloned embryos have been reported and it has been suggested that transcriptional regulation mechanism is impaired, possibly at different levels during the reprogramming process (Enright et al., 2005). Nuclear reprogramming of somatic cell nuclei in the ooplasm is a complex biological process involving the orderly, switching off and on of a diverse array of genes to support embryogenesis and concomitant developmental events (Simonsson and Gurdon, 2004).

Incomplete reactivation of embryonic developmental genes is the primary cause of low SCNT efficiency (Tsunoda and Kato, 2002). This is often related to significant up or down-regulation, de novo induction or silencing of genes for critical undisturbed fetal, and neuronal development (Giraldo et al., 2008).

Insulin-like growth factor (IGF-1), also known as somatomedin C, mediates the growth promoting activity of growth hormone. IGF-1 induces endothelial cell migration and is involved in the regulation of angiogenesis. IGF-1 exerts its action through the IGF-1 receptors. IGF-2, which is also known as multiplication stimulating activator (MSA) shows, virtually identical bioactivities similar to IGF-1 (Kim et al., 2005). IGF-2 binds to IGF-1R and IGF-2R. IGF-1 and IGF-2 are autocrine regulators of cell proliferation, paracrine growth and survival factor for mammalian embryo development (Kim et al., 2006).

The transcription of insulin-like growth factors, IGF-1, IGF-2 and their receptors, IGF-1R and IGF-2R, in granulosa cell nuclear transferred cloned embryos and in vitro fertilized babaline embryos were compared in this study.

2. Materials and methods

All chemicals used were from Sigma Chemical Co. (St. Louis, USA) unless otherwise specified.

2.1. Oocyte collection and in vitro maturation

Buffalo ovaries were obtained from a nearby abattoir and transported in normal saline (0.9% NaCl) at 25–30 °C to the laboratory within 3 h of slaughter. The ovaries were washed in normal saline and antral follicles (2–8 mm) were aspirated using an 18-gauge needle and collected into Hepes buffered tissue culture medium 199 (TCM-199) plus phosphate buffer saline (PBS; Gibco BRL/Life Technologies) supplemented with 0.1% bovine serum albumin (BSA). The cumulus oocyte complexes (COCs) showing an even cytoplasm and surrounded by at least 3 layers of compact cumulus cells were selected. COCs were washed 3 times in TCM-199 medium supplemented with 10% fetal bovine serum (Hyclone), gentamycin sulfate (10 mg/mL) and sodium pyruvate (0.2 mM). COCs were cultured in groups of 20–25 in 4-well plate (Falcon) and cultured in TCM-199 medium, supplemented with gentamycin sulfate (10 mg/mL), β-estradiol (1 μg/mL), FSH (5 μg/mL), LH (5 μg/mL) and 10% FBS at 38.5 °C in 5% CO2 in air for 24 h. Matured COCs were randomly allocated in group 10–12 per well to either IVF or SCNT.

2.2. Preparation of oocytes for nuclear transfer

Matured oocytes (after 24–26 h in maturation medium) were vortexed in solution comprising of 80 μL oocyte maturation (OM) medium (Hepes buffered TCM-199 supplemented with 10% FBS) and 20 μL hyaluronidase (0.1%) for 3 min in 1.5 mL centrifuge tubes (Eppendorf, USA) to remove the cumulus layers, and washed 5 times in the same medium. Oocytes were treated with 7.5 μg/mL cytochalasin-B (Cyto-B) in OM medium for 1 h. Oocytes at metaphase II stage showing the second polar body extruded in perivitelline space were selected for SCNT.

2.3. Donor cell preparation

Primary culture of buffalo ovarian granulosa cells was done in 25 cm² tissue culture flasks (Falcon) containing 4 mL Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, antibiotics (50 mg/L) and l-glutamine (10 mg/L) at 37 °C, 5% CO2 and 95% humidified atmosphere. At 70–80% confluency, granulosa cells were removed by trypsin/EDTA (TE) treatment and subjected to multiple passaging. To preserve them, granulosa cells were frozen with 10% dimethyl sulfoxide (DMSO) in DMEM plus 10% FBS at the fourth cell culture passage and stored in liquid nitrogen. The frozen granulosa cell vial was thawed at 37 °C and cultured in DMEM containing 10% FBS for 3–4 days and rounded cells were kept in suspension for nuclear transfer.

2.4. Production of nuclear transfer embryos

Recipient buffalo oocytes were micromanipulated according to procedure described for SCNT of goat embryos (Gupta et al., 2007) using manipulation media (TCM-199 + 10% FBS) on a Nikon Diaphot microscope equipped with Narishige micro manipulators. The zona pellucida above the first polar body was cut with a fine glass needle, and 10–15% of the ooplasm was extruded along with second polar body. The extruded droplet was placed in 5 μg/mL Hoechst 33342 and viewed in UV light for ensuring complete removal of oocyte chromatin material including polar body and metaphase II plate.

The medium sized (12–15 μm) cells were selected as nuclear donors. Individual granulosa cell of passage 5 was injected into perivitelline space of the enucleated oocyte. The cell plus oocyte complex was placed in fusion medium (0.3 M mannitol, 0.05 mM CaCl2, 0.1 mM MgSO4) followed by 5 min.
2.5. In vitro fertilization

In vitro matured COCs were fertilized with prepared sperm as previously described (Madan et al., 1994; Chauhan et al., 1998). One straw of frozen buffalo semen was thawed at 37 °C and washed twice by centrifugation at 500 × g for 10 min in Brackett and Oliphant (1975) medium (BO). After washing, the media were supplemented with 10 mM caffeine, 10 μg/mL heparin and 1 mM sodium pyruvate. The pellet was re-suspended in caffeine-BO medium at 2 × 10⁶ sperm/mL. A 50 μL aliquot of semen suspension was added to each 50 μL droplet containing oocytes, and they were co-cultured together for 6 h in CO₂ incubator at 38.5 °C. The IVF embryos were cultured at 38.5 °C in Heps buffered TCM-199 supplemented with 10% FBS medium for 7–9 days and no cell line was added as co-culture.

2.6. Differential staining

The quality of blastocyst was assessed by differential staining of the inner cell mass (ICM) and the trophoectoderm (TE) cells according to Thouas et al. (2000). Briefly, TE cells of blastocysts Day-7 were stained with 100 μg/mL propidium iodide after treatment with permeabilizing solution containing 1% (v/v) Triton X-100 ionic detergent. Blastocysts were incubated in a fixing solution containing 100% ethanol and Hoechst 33342 at 4 °C. Fixed and stained whole blastocysts were assessed for cell number using fluorescence microscopy. Blastocysts with <20 total cells were excluded from study.

2.7. Preparation of embryo lysate

Morphologically normal 2- to 16-cell, morula and blastocyst stage SCNT and IVF embryos were separately treated using a cell to cDNA II kit (Ambion, Texas, USA). Twelve embryos at each stage derived from either SCNT or IVF were used for cDNA preparation. Embryos were washed 3 times with 200 μL PBS by centrifugation for 5 min at 4 °C at 1200 × g and were lysed by adding 100 μL of lysis buffer.

2.8. DNase digestion and reverse transcription

The cell lysate was supplemented with 0.2 μL (0.4 U/μL) of DNase I, and 37 °C for 15 min immediately after incubation. Each tube was heated for 10 min at 75 °C to inactivate the DNase. To synthesize the first strand of cDNA, 5 μL cell lysate, 4 μL dNTP mix, 2 μL oligo dT and 5 μL RNase free water were assembled in a RNase-ree 0.5 mL tube, then heated Q5 for 3 min at 70 °C. After cooling the mixture on ice, 2 μL 10× RT buffer, 1 μL M-MLV reverse transcriptase and 1 μL RNase inhibitor were added to the reaction tubes. Reverse transcription was carried out for 1 h at 42 °C, followed by incubation at 95 °C for 10 min. RT minus product with all the reaction components except of the reverse transcriptase were produced for each sample, and were employed for RT-PCR in order to demonstrate that the template for the PCR product was cDNA, not genomic DNA.

2.9. Primer design

RT-PCR primers were carefully designed using the software Beacon Designer 7.0 (Premier Biosoft International, Palo Alto, CA, USA) to avoid amplification of genomic DNA. The sequence of the primers used, the fragment size, annealing temperature and the sequence references of the expected PCR products are shown in Table 2.

2.10. RT-PCR analysis of gene expression

Before the final step of gene expression analysis, each cDNA sample was first amplified with a pair of primers specific for β-actin mRNA to screen the samples for contamination with genomic DNA. RT-PCR was performed on an MX3000p Stratagene System (US) using the SYBR green qPCR SuperMix Invitrogen as a double-stranded DNA-specific fluorescent dye in 25 μL reaction to quantify the gene expression of IGF1, IGF2, IGF1R and IGF2R relative to 18S (BA259) reference in each embryo. Samples not exposed to reverse transcriptase were analyzed in triplicate for quantitative assessment of RNA amplification with PCR primers.

Final concentrations of 10 nM of forward and reverse primers and cDNA (4 μL) from one embryo equivalent along with 12.5 μL 2 × PCR SYBR Green master mix were added per reaction. Samples not exposed to reverse transcriptase (RT) were used as negative controls. The PCR conditions were

<table>
<thead>
<tr>
<th>Stage of development</th>
<th>SCNT</th>
<th>IVF</th>
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<tbody>
<tr>
<td>2-cell embryo</td>
<td>62.50 ± 2.24* (190/304)</td>
<td>79.15 ± 1.10* (843/1065)</td>
</tr>
<tr>
<td>4-cell embryo</td>
<td>88.4 ± 2.20 (168/190)</td>
<td>88.3 ± 1.70 (745/843)</td>
</tr>
<tr>
<td>8-cell embryo</td>
<td>84.5 ± 1.08 (142/168)</td>
<td>78.79 ± 0.98 (587/745)</td>
</tr>
<tr>
<td>16-cell embryo</td>
<td>91.5 ± 2.88 (130/142)</td>
<td>88.41 ± 1.04 (519/387)</td>
</tr>
<tr>
<td>Morula</td>
<td>81.5 ± 3.24 (106/130)</td>
<td>79.38 ± 1.40 (412/519)</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>64.1 ± 2.36* (68/106)</td>
<td>76.21 ± 0.84* (432/314)</td>
</tr>
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*Significance level P < 0.05.
95 °C for 10 min, then 50 cycles consisting of denaturation at 95 °C for 10 s, at an annealing temperature for 10 s and extension at 72 °C for 15 s. Subsequently, a dissociation curve analysis programme was run to confirm a single specific peak and to detect primer/dimer formation using the programme of 0 s at 95 °C, 10 s at 56 °C, and for 0 s at 95 °C with acquisition on the step mode. Product identity was confirmed by ethidium bromide-stained 2% gel electrophoresis. At least 3 sets of embryos were analyzed for each gene examined with 3 replicates for each transcript studied.

The comparative Ct method (Bustin, 2000) was used for relative quantification of target gene expression levels. Quantification was normalized to the internal control GAPDH gene. Within log-linear phase region of the amplification curve, each cycle doubled the amplified product. The ΔCt value was determined by subtracting the GAPDH Ct value for each sample from the target gene Ct value. Calculation of ΔΔCt involved using the highest sample method ΔCt as an arbitrary constant to subtract from all other ΔCt sample values. Fold changes in relative mRNA expression of the target genes were determined using the formula $2^{-\Delta\Delta C_{T}}$ (Bustin, 2000).

### 2.11. Experimental design

In experiment 1, the rates of SCNT and IVF buffalo embryos production were compared. In vitro matured oocytes showing cumulus expansions were randomly distributed (1069 for IVF and 303 for SCNT). In experiment 2, we used RT-PCR and its normalization against housekeeping GAPDH to quantify accurately IGF-1, IGF-1R, IGF-2 and IGF-2R genes in SCNT and IVF embryos at different developmental stages from the 2-cell stage to blastocysts.

### 2.12. Statistical analysis

Data of SCNT and IVF embryos were collected from 10 replicates. Rate of cleavage and embryonic development in SCNT and IVF experiments was calculated as mean ± S.D. using the SPSS statistical program (SPSS 7.5 for windows; SPSS, Inc., Chicago, IL, USA). The data were considered for significant differences by one-way ANOVA using SPSS programme ($P \leq 0.05$).

Relative quantification of target gene expression levels as fold difference was based on 3 sets of samples. Data on mRNA expression were analyzed using the light cycler software (Stratagene). After testing for normality and equal variance, one-way ANOVA followed by multiple pair wise comparisons using Tukey’s and Duncan’s multiple range tests for variable expression were analyzed using the light cycler software (Stratagene). After testing for normality and equal variance, one-way ANOVA followed by multiple pair wise comparisons using Tukey’s and Duncan’s multiple range tests for variable expression pattern between SCNT and IVF embryos at the identical developmental stage. Differences were considered to be significant at $P \leq 0.05$. If the n-fold difference relative to the calibrator for each treatment embryo equivalent to single embryo did not fall within the confidence interval for the IVF embryos, they were considered abnormal, either upregulated or downregulated.

### 3. Results

#### 3.1. Pre-implantation development of water buffalo embryos

Developmental rates of SCNT and IVF water buffalo embryos are summarized in Table 1. Stable losses were seen through embryo development for both SCNT and IVF groups. The overall cleavage rate was 62.5% (ranging from 56.8 to 64.9%) and 79.2 (ranged from 74.2 to 83.3%) in SCNT and IVF embryos, respectively. The cleaved zygotes (88.4%) from IVF embryos, respectively. The cleaved zygotes (88.4%) from IVF embryos were employed to determine the difference in the gene expression were analyzed using the light cycler software (Stratagene). After testing for normality and equal variance, one-way ANOVA followed by multiple pair wise comparisons using Tukey’s and Duncan’s multiple range tests for variable expression pattern between SCNT and IVF embryos at the identical developmental stage. Differences were considered to be significant at $P \leq 0.05$. If the n-fold difference relative to the calibrator for each treatment embryo equivalent to single embryo did not fall within the confidence interval for the IVF embryos, they were considered abnormal, either upregulated or downregulated.

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Fig. 1. Relative abundance of IGF mRNA transcripts (line) and production rates in buffalo SCNT embryos during pre (open bar). IGF-1 (A), IGF-1R (B), IGF-2 (C) and IGF-2R (D).

Fig. 2. Relative abundance of IGF mRNA transcripts (line) and production rates in buffalo IVF embryos (black bar). IGF-1 (A), IGF-1R (B), IGF-2 (C) and IGF-2R (D).
3.2. Expression of IGF mRNA transcripts

IGF-1, IGF-2, IGF-1R and IGF-2R expression levels at different stages of embryo development, i.e. at the 2-, 4-, 8-, 16-cell, morula and blastocyst stages were compared in SCNT and IVF embryos. Expression profiles are depicted by bar/line diagram (Fig. 3A–D).

IGF-1 mRNA expression in SCNT and IVF embryos is shown in Fig. 3A. Significant differences in mRNA expression were detected at 2-, 4-, 8-, 16-cell, morula and blastocyst stages. However, there were no differences in the expression profile of IVF embryos between 2- and 4-cell and between 8-cell and 16-cell stages, while the differences between these groups were significant. The mRNA expression was significantly upregulated at 2-cell stage (2.3 fold), 16-cell (2.3 fold), morula (3.6 fold) and blastocyst (5.2 fold) stages of SCNT embryos in comparison to IVF embryos, whereas at other stages there were no significant differences.

The mRNA expression IGF-1R transcript was downregulated at 4-cell stage (1.50 fold), upregulated at morula (0.7 fold) and blastocyst (0.9 fold) stages in IVF embryos as compared to SCNT embryos (Fig. 3B). Significant mRNA expression differences were detected between 2-, 4-, 8-, 16-cell, morula and blastocyst stages in both SCNT and IVF embryos. The IGF-1R expression showed decline with the increase in cell number up to 16-cell stage, then an increase and was highest in IVF blastocysts. Similar expression regulation was observed at different stages in SCNT embryos except at 4-cell stage where expression was higher (Fig. 1B).

IGF-2 expression shows that gene was activated at all stages in both SCNT and IVF embryos (Figs. 1C and 2C). From 2-cell stage, the expression level was decreased up to 8-cell stage and after that continuous increase was observed in all stages in both SCNT and IVF embryos. Fig. 3C shows that in SCNT embryos the expression was significantly higher compared to IVF embryos however, it was significantly upregulated 0.4, 0.7 and 0.9 fold at 2-cell, morula and blastocyst stages respectively, while at 16-cell stage expression was significantly downregulated (3.9 fold).

Significant differences were found in IGF-2R mRNA transcript expression at all the embryonic stages of SCNT embryos (Fig. 1D). In IVF embryos, expression level was significant at all the embryonic stages, except at the 4- and 16-cell stages (Fig. 2D). The IGF-2R mRNA transcript expression was consistently lower in SCNT embryos than in IVF embryos. Individual differences in gene expression of SCNT and IVF embryos however were significantly downregulated at 2-cell (0.2 fold), morula (0.25 fold) and blastocyst (0.1 fold) stages, while at other stages, the differences were not significant (Fig. 3D).

4. Discussion

The results on buffalo embryo development following SCNT are comparable to earlier studies in cattle (Hill et al., 2000; Edwards et al., 2001) and buffaloes (Kitiyanant et al., 2001; Simon et al., 2006). In SCNT embryos, developmental arrest was mostly at during the first cleavage stage (37.5%) and after morula stage (65.5%). Our observations agree those of Edwards et al. (2003), where >65% of one-cell cloned embryos could not develop in to morula or blastocyst stage across various species. The embryo production rates were
lower in SCNT than in IVF method, which is in agreement with earlier published data (Saikhun et al., 2004; Tong et al., 2006). In IVF embryo culture, for removing developmental block from 8- to 16-cell stage, oviduct epithelial (Fakuda et al., 1990) and cumulus (Kim et al., 1990) cell lines have been used as co-cultures. However, in SCNT embryos, co-culture with cell lines is avoided (Thompson et al., 1995; Sinclair et al., 2000) to minimize the risk of large offspring syndrome (LOS). A growing body of evidence is showing that co-culture is not required to develop viable blastocysts in vitro (Mullaart et al., 2001). To minimize the variation, we used cell line-free culture medium for both SCNT and IVF embryos.

The arrest in embryonic development has been correlated with the beginning of epigenetic alteration as shown in buffalo IVF embryos at this stage (Suteevam et al., 2006). Sharp decline in the development rate of the SCNT embryos at blastocyst stage was probably because of drastic changes in gene expression pattern at that stage. Rizos et al. (2003) held that there are differences in relative mRNA abundance of developmentally expressed genes among different species of embryos, even when embryos were cultured under the same conditions. The studies on relative gene expression of IGF and other developmental genes are lacking in water buffalo. According to Kumar et al. (2007), the aberrant epigenetic patterns suppress the pre-implantation development. Further, this aberrant behavior may cause improper expression of developmentally important genes, which in turn leads to the developmental failure of the SCNT embryos.

We compared the expression profile of growth promoting genes (IGF-1 and IGF-2) and their receptor (IGF-1R and IGF-2R) genes in SCNT cloned embryos with IVF embryos at different developmental stages. Aberrant regulation of these affected IGF components may alter specific biological processes in SCNT vis-a-vis IVF embryos. Wrenzcyki et al. (2004) reported differences in mRNA expression pattern between SCNT and IVF bovine embryos. According to Schultz et al. (1999), the main reprogramming starts after the 2-cell stage where totally differentiated cell transforms into highly differentiated totipotent blastomeres. All the bioactivities in adult tissues are generally attributed to its interaction with IGF-1 receptor; however, during embryogenesis the roles of both IGF-1R and IGF-2R may be significant in promoting normal growth (Baxter, 1988; Grimberg and Cohen, 2000). IGF receptor type-1 (IGF-1R) is a tyrosine kinase receptor, which mediates growth-stimulating effects of IGF-1 and IGF-2, such as mitogenesis, cell transformation, cell differentiation and antiapoptosis. Contrary to IGF-1R, the type 2 receptor IGF-2R, exclusively binds IGF-2 and serves for the internalization and degradation of IGF-2 (Ludwig et al., 1995).

The beneficial role of IGF-1 supplementation on in vitro buffalo embryo development is known (Pawshe et al., 1998; Mihalik et al., 2000; Purohit et al., 2005). IGF-1 mediates the growth hormone action and stimulates the growth of cultured cells (Kim et al., 2005). IGF-2 mediates growth in early mouse embryos and forms a pathway in which imprinted genes affect development during pre-implantation stages (Rappolee et al., 1992). IGF-2 ligand is imprinted when inherited maternally and IGF-2R is imprinted when inherited paternally (DeChiara et al., 1990; Barlow et al., 1991; Latham et al., 1994), consistent with the hypothesis of Haig and Graham (1991) which predicts that imprinting of growth factors, such as IGF-2, IGF-2R and IGF-1R, regulates embryonic growth in the mammalian uterus. Lighten et al. (1997) suggested that IGF-2 is parentally imprinted in human pre-implantation embryo. IGF transport and function are modulated by interaction with insulin-like growth factor binding proteins (IGFBPs) that are present in many extracellular fluids and early embryos (Winger et al., 2000; Luciano et al., 2000). However, there is little information available with regard to imprinting of these genes in buffalo embryos. Although there is no data to compare the SCNT and IVF embryos of buffalo, the variable gene expression pattern during pre-implantation development is consistent with previous work in bovines (Yaseen et al., 2001).

The expression pattern of the IGF genes analyzed was not significantly different in IVF and SCNT embryos until they passed through the early stages of segmentation. The number of gene transcripts in SCNT embryos decreased or increased in a developmentally regulated fashion, almost overlapping the profile of IVF embryos, although with significantly quantitative differences. It showed for each stage of development and gene analyzed that the RA determined for SCNT embryos is significantly different from that of IVF embryos.

The precise timing of gene activation/inactivation is crucial for a correct pre-implantation development, as delay or lack of expression leads to death of embryos (Beaujean et al., 2004). Thus, the crux of the reprogramming process is to attain the correct timing of activation and quantitative pattern of gene expression in order to initiate and continue embryonic development (Li et al., 2005). In SCNT embryos, the first major check-point of gene activation is the 2-cell stage when zygotic genome activation (ZGA) occurs. Our data show that IGF-1 and IGF-2 mRNA transcripts' expression in SCNT embryos was significantly higher than that of IVF embryos at this stage.

The presence of IGF-1 expression at different stages of pre-implantation embryo is contrary to other reports in which IGF-1 mRNA transcripts were not detected in pre-implantation IVF embryos in bovine (Yaseen et al., 2001), mice (Rappolee et al., 1992), rats (Zhang et al., 1994) and water buffalo (Daliri et al., 1999). The conflicting results with regard to IGF-1 expression in buffalo SCNT embryos may be due to differences in species, cell culture system or interaction of RT-PCR with specific components used in the experiment particularly the design of primers. This finding supports the autocrine and paracrine role of IGF-1 described earlier for bovine pre-implantation development (Nuttinck et al., 2004).

Higher number of embryonic arrest at 2-cell stage in SCNT embryos suggested that 2-cell SCNT embryo may depend to some extent on degradation of the maternal transcripts. The difference may possibly be because of their genome imprinted as still somatic or due to inappropriate epigenetic reprogramming of imprinted gene IGF-1, IGF-2 and IGF-2R, which might not have remodeled completely or correctly and had not yet assumed embryonic organization.
and functions (Morgan et al., 2005). In the majority of SCNT cloned embryos the donor cell genome may remain silent after its transfer into recipient eggs, which do not develop further. According to Rappolee et al. (1992), IGF-2 mediates growth in early embryos and forms a pathway in which imprinted genes affect development during pre-implantation stages. Changes in chromatin organization mediate promoters’ activity (Byrne et al., 2002), which is regulated by the action of enhancers that increase gene activity by several folds in IVF embryos (Nothias et al., 1995), but might not come into action in SCNT embryos.

At the 4-cell stage, the relative abundance of IGF-1 and IGF-2 transcripts was significantly different in SCNT and IVF embryos, but IGF-1R and IGF-2R transcripts were significantly higher in IVF embryos in comparison to SCNT embryos. This may be the reason for differential rate of embryonic development between SCNT and IVF procedure.

The ZGA aberrant gene expression in SCNT embryos might have been eased during the progression from the 8- to 16-cell stage and the quantitative differences between IVF and SCNT embryos were reduced for IGF-1R and IGF-2R genes. These results support the finding in bovine (Liu et al., 1997; Yaseen et al., 2001) and mouse (Tong et al., 2008) SCNT embryos.

At morula and blastocyst stages, the relative abundance of IGF-1 and IGF-2 genes was higher in SCNT embryos compared to IVF embryos, while the IGF-1R and IGF-2R mRNA transcripts were underepressed in SCNT embryos than that of IVF counterparts. Our finding is consistent with Han et al. (2003) who reported that IGF-2 expression was downregulated and IGF-2 was upregulated in cloned embryos rather than in IVF embryos (P < 0.05). This was probably because of incomplete reprogramming of the imprinted genes (IGF-1R and IGF-2R) of donor nuclei in SCNT cloned embryos as compared to IVF embryos. Similar results were reported by Daniels et al. (2000) and Amarnath et al. (2007) in SCNT cloned bovine embryos for different mRNA transcripts.

In summary, our results suggest that reprogramming of the somatic genome towards an embryonic profile of gene expression is initiated rapidly after SCNT. The somatic genome soon falls under ooplasmic control. The fact that all the genes analyzed were switched on, in the live SCNT embryos at approximately the right time compared to dead ones at a particular stage of cleavage. These results also indicate that the reprogramming phenomenon is developmentally regulated and the molecular mechanism of IGF-1, IGF-1R, IGF-2 and IGF-2R transcript expression in cloned embryos gets modified during multiple passaging through the repeated cell divisions. IGF components play a significant role in cell growth and proliferation that is ultimately reflected during development of pre-implantation of buffalo SCNT embryos. This brings a ray of hope that, by appropriate epigenetic modification of gene expression of IGF genes, this would help in improving the cloning rate. Our study on gene expression profile of IGF genes at different developmental stages provides further useful data suggesting a cell-specific pattern for the expression of IGF-transcript component in pre-implantation embryonic development. The study on more genes will also definitely help in developing molecular signatures for embryo viability in culture before their transfer in surrogate for higher success rate in buffalo cloning.

**Uncited reference**

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