Developmental expression of lineage specific genes in porcine embryos of different origins

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Abstract

Purpose This study compared the expression of genes involved in pluripotency, segregation of inner cell mass (ICM) and trophoctoderm (TE), and primitive endoderm (PE) formation in porcine embryos produced by in vitro fertilization (IVF), parthenogenetic activation (PA), and nuclear transfer (NT) using either fetal fibroblasts (FF-NT) or mesenchymal stem cells (MSC-NT).

Methods Blastocyst formation and total cell number were analyzed. The expression patterns of transcripts, including SRY-related HMG-box gene 2 (SOX2), reduced expression gene 1 (REX1/ZFP42), LIN28, caudal type homeobox 2 (CDX2), TEA domain family member 4 (TEAD4), integrin

Capsule The nuclear donor cell type influences the expression pattern of genes involved in pluripotency functions and cell fate determination in porcine NT embryos.

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beta 1 (ITGB1) and GATA6 were assessed at the 4–8 cell and blastocyst stage embryos by real-time PCR.

Results Developmental rates to blastocyst stage and total cell number were higher in IVF and PA embryos than in NT embryos. But MSC-NT embryos had increased blastocyst formation and higher total cell number compared to FF-NT embryos. The relative expressions of transcripts were higher in blastocysts than in 4–8 cell stage embryos. The mRNA expression levels of SOX2 and REX1 were largely similar in embryos of different origins. However, the genes such as LIN28, CDX2, TEAD4, ITGB1 and GATA6 showed the differential expression pattern in PA and NT embryos compared to IVF embryos. Importantly, the transcript levels in MSC-NT embryos were relatively less variable to IVF than those in FF-NT embryos.

Conclusion MSCs seem to be better donors for porcine NT as they improved the developmental competency, and influenced the expression pattern of genes quite similar with IVF embryos than that of FFs.

Keywords Gene expression · Preimplantation embryos · Nuclear transfer · Porcine

Introduction

It is well established that during mammalian preimplantation development, two sequential cell fate decisions give rise to three committed lineages; the inner layer of cells forming the inner cell mass (ICM), as well as two extraembryonic tissues, the trophoctoderm (TE) and the primitive endoderm (PE) [1, 2]. Though the major genetic and morphological transitions in the embryos of mouse, cow and pig are similar, differences persist in terms of their timing, decisions of cell fate and the allocation of lineage committed

cells [3–6]. The differences in early lineage segregation have been speculated to exert potential influences on the formation and characteristics of the pluripotent cell population. In the mouse, the process of segregation and maintenance of initial cell differentiation has been shown to be regulated by several key lineage-specific transcription factors [1, 2]. However, gene regulatory mechanisms contributing for lineage specification in porcine early stage embryos are still much less understood.

Embryonic development in pigs requires the correct temporal and spatial expression of many genes, the functions of which are critical not only for the survival of normal and viable embryos but also for the regulation of cell fate commitment and subsequent fetal and placental formation [7]. Differences in the expression pattern of pluripotency and developmentally important genes have been reported between *in vivo*- and *in vitro*-produced porcine embryos [8–11]. The inefficiency of porcine nuclear transfer (NT) has been linked with abnormal patterns of gene expression mainly due to incomplete reprogramming of donor cell genome besides aberrant epigenetic mechanisms, such as DNA methylation and histone modifications [12]. Variations in the expression profiles of genes involved in the establishment and maintenance of pluripotency, DNA methylation, histone deacetylation, apoptosis and embryonic growth have been demonstrated at various developmental stages of porcine NT embryos derived from different culture conditions [10] and donor cell types [8, 13–16]. However, very few attempts have been made to comprehensively characterize the expression of gene transcripts regulating the early lineage segregation in porcine preimplantation embryos [5]. In this study, we hypothesized that the expression pattern of genes implicated in pluripotency and differentiation of early cell lineages may indicate the reprogramming efficiency of donor cell genome by the recipient oocyte after NT.

Previous studies from our group on the developmental ability and differential gene expression patterns in porcine NT embryos demonstrated that relatively less differentiated MSCs could be better nuclear donors to produce cloned piglets [8, 17]. Extending these observations, we successfully cloned miniature pigs using MSCs as nuclear donors and proved their ability to support postimplantation development [18]. However, the overall cloning efficiency was still very low, and the possible reasons suggested to be fetal and placental abnormalities caused by faulty epigenetic reprogramming of the donor genome and aberrant patterns of gene expression in NT embryos [12]. Therefore, further characterization on the expression profile of genes during preimplantation stage was required to better understand the molecular functions regulating the cell fate and lineage specification in the early embryo. Examining the relative abundances of transcripts that regulate the segregation of ICM and TE, and formation of PE lineages will help to

reveal the extent of nuclear reprogramming in porcine NT embryos. For these reasons, in this study, we compared the expression levels of transcription factors implicated in the maintenance and regulation of pluripotency, segregation of ICM and TE, and formation of PE in porcine preimplantation embryos produced by *in vitro* fertilization (IVF), parthenogenetic activation (PA), and NT using either fetal fibroblasts (FF-NT) or mesenchymal stem cells (MSC-NT). The relative expression of transcripts, such as SRY-related HMG-box gene 2 (SOX2), reduced expression gene 1 (REX1/ZFP42), LIN28, caudal type homeobox 2 (CDX2), TEA domain family member 4 (TEAD4), integrin beta 1 (ITGB1) and GATA6 were analyzed at the 4–8 cell and blastocyst stages of embryos by real-time reverse transcription-polymerase chain reaction (real-time PCR).

Materials and methods

All experiments in the present study were conducted according to the guidelines of Animal Center for Biomedical Experimentation at Gyeongsang National University.

Supplements

Unless otherwise specified, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) and media from Gibco (Invitrogen, Burlington, ON, Canada).

Porcine oocytes preparation

Ovaries of domestic pigs were obtained from pre-pubertal gilts at a local abattoir. Collection and *in vitro* maturation (IVM) procedures for oocytes were performed as described previously [19]. Briefly, cumulus-oocyte-complexes (COCs) were aspirated from antral follicles of 3–6 mm in diameter and sets of 50 compact COCs were matured in 500 μ l droplets of tissue culture medium-199 (TCM-199) supplemented with 5 % fetal bovine serum (FBS), 0.57 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 25 mM HEPES, 2.5 mM sodium pyruvate, 1 mM L-glutamine, 0.5 μ g/ml LH and 0.5 μ g/ml FSH in each well of a four-well multi dish (Nunc, Roskilde, Denmark) for 22 h. COCs were further cultured in fresh medium without LH and FSH for 20 h at 38.5 °C in a humidified atmosphere of 5 % CO₂ in air. At 42 h of maturation, cumulus cells were removed by vortexing in Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.1 % (*W/V*) hyaluronidase for 1 min. Oocytes with a polar body (PB), intact cell membranes and even cytoplasm were selected for the production of IVF, parthenotes and NT embryos.

In vitro fertilization (IVF), parthenogenetic activation (PA) and embryo culture

For IVF, sets of 20 denuded metaphase II (MII) oocytes were transferred into fertilization droplets (50 μ L) of modified tris-buffered medium (mTBM) consisting of 113.1 mM NaCl, 3.0 mM KCl, 7.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 20.0 mM Tris crystallized free base, 11.0 mM glucose, and 5.0 mM sodium pyruvate, supplemented with 2 mM caffeine and 4 mg/ml bovine serum albumin (BSA, Fatty acid free, Fraction V). Frozen-thawed sperm prepared by Percoll (Pharmacia, Uppsala, Sweden) density gradient was added at a final concentration of 1×10^5 /ml. Following 5 h of sperm/oocyte co-incubation at 38.5 °C in a humidified atmosphere of 5 % CO_2 in air, presumptive zygotes were transferred into porcine zygote medium (PZM)-5 medium supplemented with 3 mg/ml BSA.

For parthenote embryos production, MII stage oocytes were transferred into a BTX Electro chamber (BTX, Inc., CA, USA) filled with 0.28 M mannitol solution containing 0.01 % (W/V) BSA, 0.05 μ M CaCl_2 and 0.01 μ M MgSO_4 and pulsed twice with 2.0 kV/cm DC for 30 μ s using a BTX Electro-Cell Manipulator 200 and were cultured in PZM-5 medium containing 7.5 μ g/ml cytochalasin B (CCB) for 3 h at 38.5 °C in a humidified atmosphere of 5 % CO_2 in air. Following activation, eggs were transferred into PZM-5 medium supplemented with 3 mg/ml BSA.

Embryo culture was performed by following a method previously described [20, 21]. Sets of 30 zygotes or activated eggs were cultured in 30 μ L drops of PZM-5 medium supplemented with 3 mg/ml BSA up to 7 days at 38.5 °C in a humidified atmosphere of 5 % CO_2 in air. The rates of cleavage and blastocyst development were evaluated on day 2 and day 7, respectively. For gene expression analysis, embryos at 4–8 cell and blastocyst stages were collected at 48–72 h and 168 h, respectively. All embryos were washed three times in DPBS, collected in small volumes of DPBS and stored at –80 °C until RNA extraction. For analyzing total cell numbers, day 7 blastocysts were fixed in 3.7 % formaldehyde for overnight and stained with 10 μ g/ml 4',6-diamidino-2-phenylindole solution (DAPI) for 10 min. Stained and mounted blastocysts were assessed for their nuclei under a fluorescence microscope (Leica CTR600, Heerbrugg, Switzerland).

Donor cells preparation

Porcine fetal fibroblasts (FFs) were prepared by following a method as described previously [8]. Briefly, FFs were isolated from fetuses on approximately day 30 of gestation and prepared by digestion in 0.05 % trypsin-ethylenediamine tetra acetic acid (trypsin-EDTA) for 15 min. Trypsinized cells were washed once by centrifugation at 300 $\times$ g for

10 min and subsequently cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 1 mM sodium pyruvate, 1 mM L-glutamine, 100 IU penicillin G, 100 μ g/ml streptomycin sulfate and 10 % fetal bovine serum (FBS) at 38.5 °C in a humidified atmosphere of 5 % CO_2 in air. Seeded cells were cultured to confluence for 5–7 days. For long-term storage, cells were trypsinized, frozen in DMEM supplemented with 10 % FBS and 10 % dimethyl sulfoxide and stored in liquid nitrogen. When needed, the cells were thawed and cultured in DMEM supplemented with 10 % FBS, and passages 2–5 were used for NT experiments.

Mesenchymal stem cells (MSCs) were isolated from approximately one month old domestic pig bone marrow according to the method described earlier [22], with some minor modifications. Briefly, gelatinous bone marrow extract was removed and suspended in DPBS. Mononuclear cells were isolated by density gradient centrifugation through Ficoll-Paque solution (Amersham Biosciences, Uppsala, Sweden) at 400 $\times$ g for 40 min. The cells were washed twice with DPBS and cultured in advanced-Dulbecco's modified Eagle's medium (ADMEM) supplemented with 4 mM glutamine, 100 IU penicillin G, 100 μ g/ml streptomycin and 10 % FBS, at 38.5 °C in a humidified atmosphere of 5 % CO_2 in air. Three days later, non-adherent cells were gently removed by replacing the medium. After 6–8 days of culture, adherent cells were trypsinized and subsequently passaged or stored in liquid nitrogen as described above. Cells were then re-grown under similar culture conditions and NT experiments were performed using MSCs at 2–5 passage. Isolated MSCs were characterized by flow cytometry analysis of positive [CD29 (ITGB1), CD44 and CD90] and negative (CD45) surface markers along with their in vitro differentiation capacity into adipocytes or osteocytes by following protocols as previously described [22].

Nuclear transfer (NT)

Oocytes in MII were used for the production of NT embryos and the procedure was performed according to the previously described method [19] with minor modifications. In brief, matured oocytes were placed in HEPES-buffered TCM-199 supplemented with 10 % FBS, 7.5 μ g/mL CCB and 12 mM sorbitol for enucleation. The first polar body and the adjacent small volume of cytoplasm containing MII plate were removed by aspiration with an inner diameter of 20 μ m enucleation pipette. Enucleated oocytes were stained with 5 μ g/ml bisbenzimidazole (Hoechst 33342) for 5 min. Fluorescence microscope (Nikon, Tokyo, Japan) was used to identify and select enucleated oocytes. A single intact donor cell was introduced into the perivitelline space of each recipient oocyte. Couplets were placed in a fusion chamber filled with 0.28 M mannitol solution containing 0.01 % (W/V) BSA,

0.05 μM CaCl_2 and 0.01 μM MgSO_4 . Fusion was induced by applying two successive DC pulses of 2.0 KV/cm for 30 μs duration using a BTX Electro chamber (BTX, Inc.). Fused oocytes were further incubated in PZM-5 medium containing 7.5 $\mu\text{g/ml}$ CCB for 3 h at 38.5 °C in a humidified atmosphere of 5 % CO_2 in air. Fusion of reconstructed oocytes was verified and then transferred into embryo culture media as explained above.

RNA extraction and complementary DNA (cDNA) preparation

Donor cells (FFs and MSCs), and pools of ten to thirty 4–8 cell and blastocyst stage IVF, PA, FF-NT and MSC-NT embryos (triplicates) were used. Total RNA was extracted with RNeasy® MicroKit (Qiagen, Hilden, Germany) following the manufacturer's protocol and including DNase I treatment to remove genomic DNA. RNA was eluted in a final volume of 14 μl of RNase-free water. Complementary DNA (cDNA) was synthesized at 37 °C for 60 min using Omniscript reverse transcription Kit (Qiagen) with oligo-dT primers. cDNA samples with a final volume of 20 μl were stored at –20 °C until further use.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR

RT-PCR and real-time PCR was carried out for gene transcripts, such as SOX2, REX1/ZFP42, LIN28, CDX2, TEAD4, ITGB1 and GATA6. RT-PCR was performed to confirm the expression of selected transcripts and their product size in donor cells and embryos. PCR amplification was carried out in a PTC-200 Peltier thermal cycler (MJ Research Inc. MA, USA) using the Maxime-PCR PreMix Kit (iNtRON BIO., Sungnam, Korea) in 30 or 35 cycles with each cycle consisting of initial denaturation step at 94 °C for 30 s, annealing step at 55–62 °C for 30 s and elongation step at 72 °C for 90 s and 10 min final extension at 72 °C. The PCR products were subjected to electrophoresis on a 1 % agarose gel. Details on each specific gene used for PCR are summarized in Table 1. Real-time PCR was carried out on a LightCycler® using FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. The real-time PCR mix consisted of 2 μl of cDNA, 2 μl of the FastStart DNA Master SYBR Green I reaction mix, 3 mM MgCl_2 , and 1 μl each of the forward and reverse primer and was adjusted to a total volume of 20 μl using nuclease-free water. The amplification conditions consisted of initial denaturation at 95 °C for 10 min followed by 40 cycles of PCR with denaturation at 95 °C for 10 s, annealing at 55 °C–62 °C for 5 s and extension at 72 °C for 10 s. Fluorescence was acquired in each cycle to determine the crossing point

(CP) or the cycle during the log-linear phase of the reaction at which fluorescence of a sample rises above background fluorescence. The specificity of PCR products obtained was confirmed by melting curve analysis and PCR product sizes were verified by agarose gel electrophoresis. Embryos were analyzed in triplicates for all transcripts and the relative quantification of mRNA was determined by the comparative $2^{-\Delta\Delta\text{CP}}$ method [PE Applied Biosystems, Perkin Elmer, Foster City, CA, [23]]. During real-time amplification, the calibrator consisted of cDNA from MII oocytes. The results were reported as the relative expression after normalization of the amount of transcript to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an internal control gene in each cDNA sample. GAPDH was used as a house-keeping gene, as previously demonstrated [24].

Statistical analysis

All data were obtained from a minimum of 3 replicates. The proportional data were subjected to an arc-sine transformation to meet normality assumptions, and one-way Analysis of Variance (ANOVA) was used to determine the statistical significance among the treatments (PASW statistics 18, SPSS Inc.). The comparisons of mean values among treatments were made by Bonferroni or Tukey's multiple comparisons test. A P-value of <0.05 denoted a significant difference. Data are expressed as mean $\pm$ standard deviation (SD).

Results

General observations

Bone marrow derived MSCs were successfully attached on cell culture plates with fibroblast-like morphology, maintained proliferation and could be easily subcultured after dissociation with trypsin-EDTA treatment. We then performed FACS analysis to test their surface markers expression (Fig. 1a). The cultured cells between passages 2 and 5 were trypsinized and harvested for analysis. Cells positively expressed CD29, CD44 and CD90, which were widely considered as positive markers of MSCs. In contrast, CD45, a well known hematopoietic cell marker was barely detectable. Further, MSCs cultured in adipogenic medium were positively stained by Oil red O indicating the formation of oil droplets (Fig. 1bI). After treatment with osteogenic induction medium, the evidence of calcium deposition was observed by von Kossa staining (Fig. 1bII). However, MSCs remained as fibroblast-like cells in untreated controls (Fig. 1bIII). Collectively, these results confirmed that the isolated cells comprised the MSC population.

Table 1 Primers used for RT-PCR and real-time PCR

Gene	Sequence	Length (bp)	Accn. No./ reference
SOX2	F-GCCTGGGCGCCGAGTGGA R-GGGCGAGCCGTTTCATGTAGGTCTG	443	NM_003106 [45]
REX1	F-TTTCTGAGTACGTGCCAGGCAA R-GAACGGAGACACGCTTCTCAGAG	201	TC206552 [33]
LIN28	F-TTGGGAGCGAGAGACGGCCC R-GCTGGGCCTTCAGTGGACACG	186	NM_001123133.1
CDX2	F-GTCACCAGAGCTTCTCTGGG R-AGACCAACAACCCAAACAGC	144	EU137688 [5]
TEAD4	F-AGCGGAGCCTTGGAGGGCAA R-ATGATCTTGCGCCGACCACA	209	NM_001142666.1
ITGB1	F-TGAGTGTGGGGCCTGCAGGT R-GTCACAGCGCTGCCAGTGT	322	NM_213968.1
GATA6	F-TGCAGTCCAGACTGGGCCGA R-TGGCCCCGGTCTTAGTCCCG	298	NM_214328.1
GAPDH	F-GGGCATGAACCATGAGAAGT R-AAGCAGGGATGATGTTCTGG	230	AF017079

Developmental rates of porcine preimplantation embryos of different origins

The developmental rates to the cleavage (day 2) and blastocyst (day 7) stages were determined in IVF, PA, FF-NT and MSC-NT embryos, and the results are summarized in Table 2. IVF, PA and NT embryos demonstrated a different pattern of development. The developmental rates for cleaved embryos derived from FF-NT and MSC-NT (57.48 ± 7.49 and 59.12 ± 3.10 , respectively) were significantly ($P < 0.05$) lower than those for embryos derived from IVF and PA (86.69 ± 2.94 and 87.98 ± 2.46 , respectively). Further, significantly

($P < 0.05$) higher rates of blastocyst formation were observed in IVF (31.90 ± 3.38) and PA (33.60 ± 3.77) embryos. However, the rates of porcine cloned embryos that developed to the blastocyst stage were significantly ($P < 0.05$) higher in MSC-NT embryos (17.82 ± 4.37) than that of FF-NT embryos (10.53 ± 3.18).

Total cell number in porcine preimplantation embryos of different origins

Figure 3 shows the total cell number in porcine day 7 blastocysts derived from IVF, PA and NT (FF and MSC)

Fig. 1 Characterization of porcine bone marrow derived MSCs. **a.** Representative images showing flow cytometric analysis of cell surface markers CD29, CD44, CD90 and CD45. Filled histograms represent staining with specific antibodies and open histograms correspond to matched isotypes. **b.** Adipogenic differentiation was indicated by

intracellular lipid accumulation with Oil red O staining (I). Osteogenic induction was visualized by detection of dark calcium deposits after von Kossa staining (II). Untreated MSCs remained as fibroblast-like cells. Scale bar=50 μ m (I), 100 μ m (II), 250 μ m (III)

Table 2 Developmental potential and total cell number in porcine IVF, PA, FF-NT and MSC-NT embryos

Groups	No. of embryos cultured	No. of embryos cleaved (mean %±SD)	No. of embryos developed to blastocyst (mean %±SD)	Total cell number, mean %±SD (Number of embryos counted)
IVF	298	258 (86.69±2.94) ^b	95 (31.90±3.38) ^c	60.4±7.23 ^c (8)
PA	301	265 (87.98±2.46) ^b	101 (33.60±3.77) ^c	51.2±5.54 ^c (8)
FF-NT	357	205 (57.48±7.49) ^a	37 (10.53±3.18) ^a	26.8±3.42 ^a (7)
MSC-NT	304	180 (59.12±3.10) ^a	54 (17.82±4.37) ^b	39.2±5.12 ^b (8)

Different superscripts (^{a–c}) in the same column indicate a significant difference ($P<0.05$). Five replicates

embryos. Overall, a significantly ($P<0.05$) higher total cell number was observed in IVF and PA embryos when compared with FF-NT and MSC-NT embryos (Table 2, Fig. 2a–IV and b). The mean total cell number of IVF and PA embryos did not reveal any significant ($P>0.05$) differences (60.4±7.23 and 51.2±5.54, respectively). However, the mean total cell number of NT embryos from MSCs (39.2±

5.12) were significantly ($P<0.05$) higher than that of FFs (26.8±3.42).

Detection and relative expressions of gene transcripts in porcine preimplantation embryos of different origins

The composite images of bands of RT-PCR amplification products and relative expression levels by real-time PCR represent the results of gene transcripts analysis involved in pluripotency, segregation of ICM and TE, and PE in porcine embryos produced by IVF, PA, and NT using either FFs or MSCs (Fig. 3b). The expression patterns of transcripts, including SOX2, REX1, LIN28, CDX2, TEAD4, ITGB1 and GATA6 were assessed at 4–8 cell and blastocyst stage embryos. As analyzed by RT-PCR, all selected gene transcripts showed their anticipated product sizes, and they were clearly detected in the early developmental stages of porcine embryos derived from different origins. Further, the possibility of the donor cells that already express the selected genes was examined. None of the selected gene transcripts was detected in FFs, whereas, positive expressions of SOX2, ITGB1 and GATA6 transcripts were observed in MSCs (Fig. 3a).

The relative expression level of the pluripotency regulator gene SOX2 exhibited a similar pattern in IVF, PA and NT embryos. There were no significant ($P>0.05$) differences in transcription levels of SOX2 at the 4–8 cell or blastocyst stages among the embryos of different origins. However, low levels of this gene were observed at the 4–8 cell stage embryos than in blastocysts of IVF, PA and NT. Interestingly, another pluripotency related gene REX1 followed a statistically similar expression pattern of SOX2 transcript, except its significantly ($P<0.05$) higher level in IVF than in PA and NT embryos at the 4–8 cell stage. Although the expression levels of REX1 in IVF and MSC-NT blastocysts were tended to be higher than in PA and FF-NT, the differences did not reach statistical significance. The expression of LIN28 at the 4–8 cell stage IVF embryos showed significantly ($P<0.05$) higher levels than PA, FF-NT and MSC-NT embryos. At the blastocyst stage, its mRNA levels were significantly ($P<0.05$) higher in IVF,

Fig. 2 Analysis of total cell number in porcine embryos of different origins. **a.** Representative images showing the total cell number in IVF (I), PA (II), FF-NT (III) and MSC-NT (IV) blastocysts. Nuclei were labeled with DAPI (blue) for counting of cell number. Scale bar= 50 μM. **b.** A bar graph showing the mean total cell number in IVF, PA, FF-NT and MSC-NT blastocysts. Different superscripts (a, b, c) indicate significant differences between the groups ($P<0.05$)

Fig. 3 RT-PCR and real-time PCR analysis showing gene expression profile of porcine donor cells, and embryos derived from IVF, PA, FF-NT and MSC-NT. **a.** Expression of SRY-related HMG-box gene 2 (SOX2), reduced expression 1 (REX1/ZFP42), LIN28, caudal type homeobox 2 (CDX2), TEA domain family member 4 (TEAD4), integrin beta 1 (ITGB1) and GATA6 transcripts in donor cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. **b.** Relative expression of selected genes at 4–8 cell and

blastocyst stages of embryos. GAPDH was employed as a housekeeping gene for normalization. Data is the mean $\pm$ SD. Different superscripts (a, b, c) represent significant differences in relative expression between IVF, PA, FF-NT and MSC-NT embryos at a given stage of development ($P < 0.05$, ANOVA, followed by Bonferroni or Tukey test for post-hoc comparisons). Representative RT-PCR gel photographs of the respective gene transcript in IVF, PA, FF-NT and MSC-NT embryos at 4–8 cell and blastocyst stages are presented at the bottom

but there was no difference compared to MSC-NT embryos. Further, no significant ($P > 0.05$) differences in the relative expressions of LIN28 mRNA were observed between PA and FF-NT blastocysts.

The CDX2 transcript level at the 4–8 cell stage embryos was significantly ($P < 0.05$) higher in FF-NT than in IVF, PA and MSC-NT groups. However, at the blastocyst stage, the relative abundance of CDX2 was significantly ($P < 0.05$) higher in IVF than in embryos of other origins. Regardless of differences in embryo origin, the expression level of CDX2 did not vary among PA, FF-NT and MSC-NT blastocysts. In the case of TEAD4, the expression level at the 4–8 cell stage was comparatively low in PA, FF-NT and MSC-NT embryos. Although its level was higher in IVF at the same developmental stage, no significant difference was observed with MSC-NT embryos. TEAD4 expression was markedly upregulated in the blastocysts of IVF and the levels were significantly ($P < 0.05$) higher than that of MSC-NT, FF-NT and PA. Further, the higher TEAD4 transcript level was observed in MSC-NT blastocysts compared to FF-NT and PA derived blastocysts. The mRNA expressions of ITGB1 at the 4–8 cell stage embryos showed statistically similar levels among different groups. The levels of ITGB1 transcript were significantly ($P < 0.05$) higher in MSC-NT blastocysts, but no differences were found compared to IVF blastocysts. Moreover, there were no significant ($P > 0.05$) differences in the relative expression levels of ITGB1 transcript among IVF, PA and FF-NT blastocysts.

Differences in the transcript abundance for GATA6 were observed at the 4–8 cell stage among IVF, PA and NT

embryos. The relative expression was higher in IVF group, but no significant ($P > 0.05$) difference was observed compared to MSC-NT embryos. Further, a statistically similar expression was observed among the 4–8 cell stage PA, FF-NT and MSC-NT embryos. GATA6 showed the increase in mRNA abundance at the blastocyst stage of IVF and MSC-NT embryos, and the expression levels were significantly ($P < 0.05$) higher than that of PA and FF-NT blastocysts.

Discussion

In the current study, we employed somatic cells (FFs) and tissue-specific multipotent stem cells (bone marrow derived MSCs) as nuclear donors for porcine cloning experiments, and compared the in vitro developmental potential, and the mRNA expression profile of SOX2, REX1, LIN28, CDX2, TEAD4, ITGB1 and GATA6 at the 4–8 cell and blastocyst stages of embryos with that of IVF and PA groups. Firstly, MSCs were obtained from whole bone marrow aspirates and their stem cell characteristics were established under in vitro culture conditions. Freshly isolated MSCs showed a characteristic property of adherence to plastic culture dishes and constituted a rapidly expanding cell population. Isolated cells exhibited fibroblast-like cell growth, and positively expressed MSC specific surface markers, such as CD29, CD44, CD90, but lacked the expression of hematopoietic lineage marker CD45. Furthermore, MSCs were successfully differentiated in vitro into adipocytes and osteocytes.

Taken together, isolated cells showed characteristic properties of porcine MSCs that have been well documented by previous reports [8, 18, 22, 25, 26].

In the present study, IVF and PA embryos showed higher cleavage and blastocyst rates, and total cell number than NT embryos from FFs and MSCs. However, MSC-NT embryos showed enhanced developmental potential and total cell number when compared with FF-NT embryos. The finding that the use of MSCs as nuclear donors improved early developmental competence is consistent with our earlier observations and also previous studies by others [8, 17, 18, 25–27]. Porcine cloned embryos derived from undifferentiated MSCs and their mesenchymal lineage derivatives resulted in superior preimplantation development compared to fetal or adult fibroblasts [18, 25]. Furthermore, NT embryos reconstructed with MSCs showed increased total cell number and ICM ratio, and low apoptotic positive cells when compared to fibroblasts [17]. Recently, viable cloned offspring were also produced after the transfer of porcine embryos reconstructed with undifferentiated MSCs [18] or Trichostatin A-treated blastocysts derived from putative MSCs [27]. Thus, MSCs showed a greater potential as nuclear donors than skin fibroblasts, and possessed the ability in supporting the preimplantation development of cloned pig embryos to term.

Impaired developmental competency of porcine cloned embryos has been correlated with drastic changes in gene expression pattern at the preimplantation stage [8, 13–15]. Recently, we found an aberrant expression pattern in NT-derived embryos with respect to genes involved in the establishment and maintenance of pluripotency, DNA methylation, histone deacetylation, apoptosis and embryonic growth [8]. Further, more differentiated somatic donor nuclei were shown to increase the incidence of altered gene expression profile. Extending these findings, in this study, no differences were found among the NT-derived embryos using either FFs or MSCs as donor cells with regard to expression levels of SOX2 and REX1 transcripts. Moreover, CDX2 mRNA showed statistically similar levels between FF- and MSC-NT derived blastocysts. However, the mRNA abundances of LIN28, TEAD4, ITGB1 and GATA6 transcripts were relatively higher in NT-derived blastocysts from MSCs than those from FFs. In addition, the expression pattern of selected lineage-specific transcription factors in MSC-NT embryos was more comparable to IVF embryos than that of FF-NT embryos. These results reconfirm that reprogramming was more efficient for the MSC genome than the fibroblast genome, and provide further evidence on the relationship between donor cell differentiation status and nuclear transfer success. This is supported by the fact that, the expression of SOX2, ITGB1 and GATA6 was positively detected in MSCs, indicating that these cells could be easily remodeled to embryonic state by porcine

oocytes. Our data also revealed that a few genes were differentially expressed in PA embryos to that of IVF embryos, which is not unexpected in view of previous observations [9].

In pigs, recent studies reported the presence of autoregulatory circuit that establishes and maintains the pluripotent state in epiblast cells through the co-expression of OCT4, together with NANOG and SOX2 [11, 28]. The aberrant reactivation of these fundamental regulatory genes was suggested to contribute primarily to the compromised development of cloned embryos [8, 13, 15]. Like OCT4 gene, SOX2 transcript was present throughout the porcine embryo development [9], and showed the tendency of decreased abundance at the blastocyst stage in IVF and NT embryos [15]. In contrast to these findings [15], we found the SOX2 transcript at slightly higher levels in blastocysts than the 4–8 cell stage embryos. However, the relative expression of SOX2 was similar between IVF and NT embryos at different developmental stages examined [15], and our data are consistent with this observation. Evidences indicate that SOX2 encodes transcription factors with a single high mobility group (HMG) DNA-binding domain and acts cooperatively with OCT4 to maintain pluripotency [29], and interacts with NANOG in regulating transcription of REX1 [30]. REX1 or ZFP42 is another well known marker of the ICM and expressed during porcine embryogenesis [14, 31], epiblast cells [32] and endoderm cell lines derived from blastocysts [33]. It has also been shown that REX1 is indeed controlled by the trio of OCT4, SOX2 and NANOG network [30]. This circuitry is further characterized by the association of another pluripotency factor, LIN28, which modulates OCT4 expression at the post-transcriptional level [34]. Cumulative findings suggest that SOX2, REX1 and LIN28 are required to maintain the developmental potential of embryos and correctly mark the cell lineages of the blastocysts. In the present study, expression levels of LIN28 were tended to be slightly higher in IVF and MSC-NT blastocysts, though not significant among different embryo groups for SOX2 and REX1. Interestingly, similarities in the expression of SOX2 and REX1 transcripts did not correspond with the differences in developmental rates or number of cells observed among the blastocysts of different embryo origins. The precise reason for this functional inconsistency remains unclear and needs to be further investigated. But our results are in agreement with previous findings that demonstrated lower levels of SOX2 and REX1 in embryos produced by IVF or NT [9, 14, 15]. In addition, elevated mRNA expression of SOX2 in porcine day 11 [28], day 9.5 and 10.5 embryos [11], and REX1 in endoderm cell lines of day 11 blastocysts indicated that these transcripts may have a lesser prominent role in defining the pluripotent cell population in the blastocysts of earlier stages. Furthermore, as suggested earlier, the above findings also reveal a less actively

regulated state of pluripotency in the porcine early blastocysts compared to epiblasts [28].

In mouse preimplantation embryos, the transcription factor CDX2 has been proved to be essential for the formation and maintenance of TE lineage cells [35, 36]. Studies have also showed that the reciprocal expression of CDX2 and OCT4 contributes to the commitment and establishment of TE and ICM lineages [35, 37]. However, unlike in mouse expanded blastocysts, the OCT4 expression was not restricted to ICM exclusively in porcine blastocysts, but expressed in both ICM and TE, indicating the divergence of regulatory circuitry in determining ICM/TE identity between species [4, 5, 28]. Whereas, the involvement of CDX2 in the segregation of TE was clearly demonstrated in porcine blastocysts by showing its lineage restricted mRNA and protein expression pattern [5]. Further, the expression of CDX2 was detectable in preimplantation porcine embryos [5, 14, 31] and observed at higher levels in the blastocysts compared to earlier developmental stages [5]. In accordance with these results, we were able to detect the CDX2 transcript at the 4–8 cell and blastocyst stage embryos of different origins and also, elevated levels in IVF blastocysts. However, relative transcript levels of CDX2 were lower, but significantly not different among PA, FF-NT and MSC-NT blastocysts. In contrast, an earlier study showed the CDX2 mRNA at statistically similar levels between IVF and NT blastocysts [14], and these results were correlated with no differences on the blastocyst rates. Therefore, inadequate levels of TE transcription factor CDX2 at the blastocysts stage of NT-derived embryos in this study indicate their decreased developmental competence and insufficient cell numbers. Even if embryos eventually grow further, chances of normal development in the advanced stages are limited. But considering the dynamic changes of gene expression during embryonic development, further analysis of CDX2 transcript level in individual NT-derived embryos is required.

Recently, TEAD4 expression has been observed in the specification of the TE lineage by activating the upstream of CDX2 and eomesodermin (EOMES), and direct regulation of the TE formation in mice [38, 39]. Moreover, it has been suggested that GATA3 and CDX2 act in parallel pathways, and rely on TEAD4 transcript to induce the expression of target genes within the TE lineage [40]. These reports reveal a critical requirement of TEAD4 in the blastocyst for the initiation of TE formation and for specific activation of CDX2 expression. However, the expression of TEAD4 transcript has not been investigated in porcine embryos till date. In addition, the temporal and spatial expression pattern of the membrane receptor protein ITGB1 has also been described in cell adhesion and recognition in a variety of embryonic processes including trophoblastic function [41]. In the present study, both TEAD4 and ITGB1 genes were detected at the 4–8 cell and blastocyst stages of embryos derived from IVF, PA

and NT. Relative expression levels of TEAD4 were higher in IVF embryos followed by MSC-NT, FF-NT and PA groups. In contrast, transcript level of ITGB1 was higher in MSC-NT blastocysts with no statistical difference compared to IVF blastocysts. Importantly, at the blastocyst stage, higher abundances of both TEAD4 and ITGB1 transcripts were observed in NT embryos derived from MSCs than from FFs. In recent studies, the mRNA expressions of TEAD4 and ITGB1 were detected at higher levels in *in vivo* derived bovine and porcine blastocysts, respectively compared to IVF and NT-derived blastocysts [10, 42]. Though the protein levels of TEAD4 and ITGB1 markers remain to be elucidated, the amount of transcripts observed in the blastocysts underscores the participatory role in TE lineage formation. Therefore, it may be reasonable to suggest that relatively higher expression of TEAD4 and ITGB1 is necessary for the precise regulation of the cell fate of extra-embryonic lineage.

GATA6 is a zinc-finger transcription factor essential for the development of the primitive and extraembryonic endoderm in mouse embryogenesis [43, 44]. As a common marker of the PE lineage, GATA6 protein was expressed in the ICM of *in vivo*-produced porcine blastocysts, but was absent in *in vitro*-produced embryos possibly as a result of differences in embryo origin and/or the developmental age [5]. However, the expression of GATA6 transcript was significantly higher in blastocysts than at earlier stages, and indicated its involvement in establishing the PE in pigs. In this study, GATA6 showed the upregulation in the IVF and MSC-NT blastocysts, and the expression levels were significantly higher than that of PA and FF-NT blastocysts. Despite the expression of GATA6, the high degree of variability in the transcript level observed in FF-NT blastocysts suggests that the reprogramming of the donor cell genome was incomplete and inappropriate.

In summary, the type of donor cell influenced the expression pattern of genes critical for the regulation of pluripotency and specification of the initial cell differentiation as well as development of extra-embryonic lineage in porcine cloned embryos. For all the genes analyzed here, the genes such as LIN28, TEAD4, ITGB1 and GATA6 demonstrated a low level of mRNA at the blastocyst stage in PA and FF-NT compared with IVF and MSC-NT embryos. Further, the mRNA levels of these genes were low at the 4–8 cell stage and did not increase significantly at the blastocyst stage. The outcome of less aberrant gene expression in MSC-NT embryos is most likely related to the reprogramming of MSCs with high efficiency following NT. These findings reconfirm that multipotent MSCs influence the success of cloning through improved nuclear reprogramming and transcriptional changes, and serve as better donors for porcine NT.

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Authors contribution Conceived and designed the experiments: BMK, GHM, GJR. Performed the experiments: BMK, GHM, RHJ, WJL. Analyzed the data: BMK, YML, BGJ. Contributed reagents/materials/analysis tools: SAO, GJR. Wrote the paper: BMK, BGJ, GJR.

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