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Original Article

Blastocysts derivation from somatic cell fusion with premature oocytes (prematuration somatic cell fusion)

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This study was undertaken to investigate the development of immature oocytes after their fusion with male somatic cells expressing red fluorescence protein (RFP). RFP-expressing cells were fused with immature oocytes, matured *in vitro* and then parthenogenetically activated. Somatic nuclei showed spindle formation, 1st polar body extrusion after *in vitro* maturation and protruded the 2nd polar body after parthenogenetic activation. RFP was expressed in the resultant embryos; two-cell stage and blastocysts. Chromosomal analysis showed aneuploidy in 81.82% of the resulting blastocysts while 18.18% of the resulting blastocysts were diploid. Among eight RFP-expressing blastocysts, Xist mRNAs was detected in six while Sry mRNA was detected in only one blastocyst. We propose "prematuration somatic cell fusion" as an approach to generate embryos using somatic cells instead of spermatozoa. The current approach, if improved, would assist production of embryos for couples where the male partner is sterile, however, genetic and chromosomal analysis of the resultant embryos are required before transfer to the mothers.

Key words: aneuploidy, haploidization, immature oocyte, infertility, sterility.

Introduction

The oocyte is the only cell of the body that can reprogram transplanted somatic nuclei as it contains a set of proteins, RNAs, lipids and small molecules that enable such reprogramming (Pfeiffer *et al.* 2011). The oocyte has the ability to: (i) reprogram the injected somatic cells even when it is enucleated as in somatic cell nuclear transfer (cloning) (Wilmut *et al.* 1997); (ii) enforce the somatic cell nuclei to bypass an obligatory mitotic cell cycle checkpoints and undergo premature condensation (Szollosi *et al.* 1986); and (iii) enforce the somatic cell nuclei for entry of meiosis through the reduction division as known by semi-cloning or haploidization (Takeuchi *et al.* 2001; Tesarik *et al.* 2001, Eichenlaub-Ritter 2003, Tesarik & Mendoza 2003). Moreover, it was found that the early meiotic ooplasm was able to induce initiation of a meiosis-like reducing division in mitotic nuclei originating from differentiated somatic cells (Kubelka & Moor 1997). Moreover, Salamone *et al.* revealed that haploidization of somatic cells can be induced by germinal vesicle (GV) ooplasm (Salamone *et al.* 2001, 2002). Thus, the meiotic machinery property of the ooplasm can be helpful for generation of artificial oocytes or male germ cells through injecting male and/or female somatic nuclei into enucleated mature or immature oocytes (Tsai *et al.* 2000; Trounson 2001; Tesarik 2002).

Some attempts to fuse somatic cells with the oocytes as an alternative for germ cells for fertilization have been reported; Fulka *et al.* 2002 found that somatic cells were not able to proceed through the reduction division (haploidization) in the meiotic cytoplasm due to chromosomes incompatibility between the oocytes and the somatic cells. Another study

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showed that blastocysts formation was very limited (<4%) after cumulus cell injection into non-enucleated mature oocytes because of abnormalities in the spindle formation (Fujii & Funahashi 2008). Another study reported a successful haploidization after somatic cell injection into mature oocytes which were subsequently activated (Lacham-Kaplan *et al.* 2001). All of these previous studies were based on morphological assessment and have not tracked whether the resulted blastocysts were specifically derived from the injected somatic cells or not, i.e. either through chromosome analysis and/or exclusion of parthenogenesis after artificial activation.

Fluorescent protein markers provide unprecedented insights into visualizing and tracking the incorporation of the genetic materials and its correlation with the functioning of the translated proteins (Lippincott-Schwartz & Patterson 2003).

Therefore, the current study was carried out to investigate the development of immature oocytes after their injection and fusion with male somatic cells expressing red fluorescence protein; their maturation *in vitro*; their parthenogenetic activation and subsequent early embryonic development.

Materials and methods

Chemicals

All chemicals were obtained from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA) unless otherwise stated.

Ovaries and cumulus-oocyte complexes recovery

Ovaries were obtained from sows and gilts at a local slaughterhouse and were transported to the laboratory in 0.9% NaCl at 25–30°C. Follicular fluid including cumulus-oocyte complexes (COCs) were aspirated from antral follicles (3–6 mm in diameter) and washed three times with tissue culture medium (TCM)-199-HEPES (Invitrogen, Carlsbad, CA, USA) and selected for subsequent *in vitro* maturation (IVM) on the basis of morphological features, i.e. a compact multi-layered cumulus mass and a dark, evenly granulated cytoplasm. Immediately after collection, oocytes and cumulus cells were separated by pipetting with 0.1% hyaluronidase in Tyrode's albumin lactate pyruvate (TALP). Immature oocytes were transferred to TALP microdrops overlaid with mineral oil in an atmosphere of 38.5°C and 5% CO_2 for 20 min. Cumulus-free oocytes with intact cell membrane and homogenous cytoplasm were selected under stereomicroscope for donor cell injection and subsequent experiments.

Donor cell preparation

Sinclair male skin fibroblasts (121 d) with diploid chromosomes number were used as donor cells. The cells were confirmed to express red fluorescence protein (RFP) as shown in Figure 1A. Information about the vector (pCMV_RFP-P2A-hSNCA) is shown in Figure S1. Genomic DNA of donor cells were examined for RFP DNA integration through polymerase chain reaction (PCR) using specific primers as explained later.

Donor cell injection, fusion and in vitro *maturation of injected oocytes*

Red fluorescence protein-expressing cells were selected after brief exposure to UV rays and each cell was deposited into the perivitelline space of the immature oocytes in a TALP medium containing cytochalasin B (5 μg/mL) then were washed two times with TALP. The couplets were then placed in a fusion medium comprising 0.26 mol/L mannitol, 0.1 mmol/L $MgSO_4$, 0.5 mmol/L HEPES and 0.05% bovine serum albumin (BSA) and were transferred in between cell fusion electrode needles (BTX Electro cell manipulator, BTX, San Diego, CA, USA). Fusion was induced by single DC pulse of 1.75–1.85 kV/cm for 30 μs using BTX Electro cell manipulator (Kim *et al.* 2014). Oocytes were then cultured in PZM5 medium microdrops in a humidified atmosphere of 5% CO_2 at 38°C. Fusion of the donor cells and oocytes was observed 1 h after electric stimulation under a stereomicroscope. Only fused oocytes were selected for *in vitro* maturation which was aided by co-culture with intact COCs in four-well dishes (25 injected oocytes + 15 COCs per well; Falcon, Becton Dickinson Ltd, Plymouth, UK) in basic maturation medium, TCM-199 supplemented with 10 ng/mL epidermal growth factor (EGF), 0.57 mmol/L cysteine, 0.91 mmol/L sodium pyruvate, 5 μg/mL insulin, 1 μg/mL Follicle stimulating hormone (FSH) (Antrin, Teikoku, Japan) and 1% (v/v) Pen-Strep (Invitrogen) at 38°C in a humidified atmosphere of 5% CO_2 for 44 h (two stages, 22 h each with hormonal removal in the second stage).

Parthenogenetic activation and in vitro *culture of matured oocytes*

After 44 h, the injected oocytes were selected by glass capillary pipettes and pipetted in 0.1% hyaluronidase in TALP to remove the attached cumulus cells and then were sequentially equilibrated in a gradient concentration

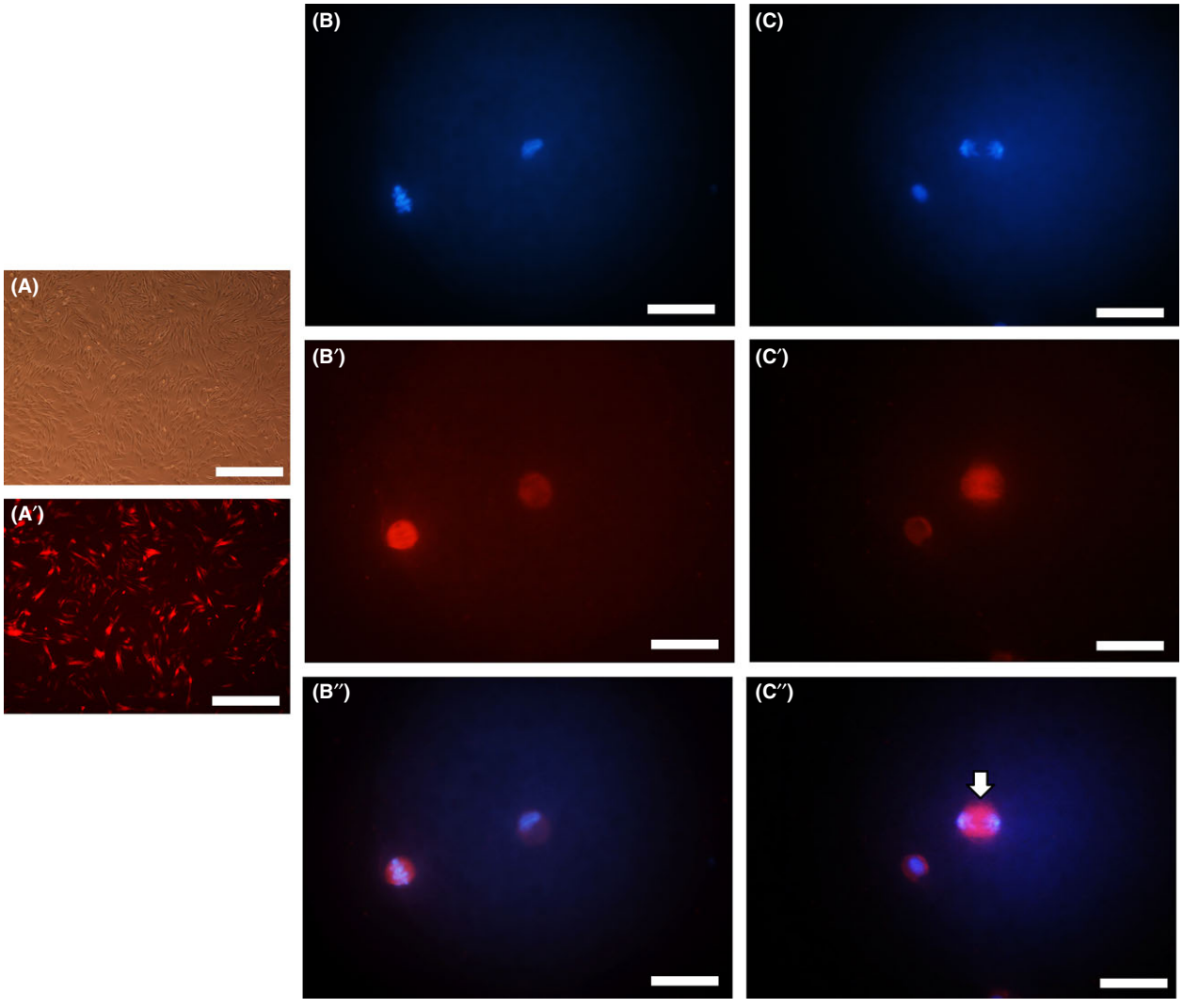

Fig. 1. Representative images of red fluorescent protein (RFP)-expressing somatic cells after 18 h from fusion with premature oocytes. A and A′ represent the cultured RFP-expressing boar fibroblasts in bright field microscope and after UV rays exposure, respectively, scale bar = 100 μm. B, Hoechst stain B′, α-tubulin B″, merge immunofluorescence localization of spindle formation (α-tubulin; red) around the nuclear material (blue) indicating the metaphase I stage for both oocyte and injected somatic cell. C, Hoechst stain C′, α-tubulin C″, merge immunofluorescence localization of α-tubulin with division of one nucleus into two portions indicating the anaphase I (arrow). Scale bar = 20 μm.

(0%, 33%, 66% and 100%) of mannitol solution (the composition is as mentioned above in addition to 0.1 mmol/L $CaCl_2$) in a four-well dish. The oocytes were then transferred into a two electrodes chamber connected with a BTX Electro cell manipulator and activated by a single pulse of 1.5 kV/cm for 60 μs and were kept for 3 min in the activation medium (Kwon *et al.* 2014). Electrically activated embryos were equilibrated in reverse order to pre-activation to decrease the stress on the oocytes. Activated oocytes were then washed in TALP and then in the culture medium and randomly distributed to the designed group for *in vitro* culture (IVC). Embryo density was adjusted to be one embryo/1 μL culture medium. Culture medium was serum free chemically defined porcine zygote medium-5 (PZM-5) in microdrops culture system (Funakoshi Co., Tokyo, Japan) covered with mineral oil in an atmosphere of 38.5°C, 5% CO_2, 5% O_2 and 90% N_2. Blastocyst formation was assessed on days 5–7. The process of somatic cell injection, fusion, *in vitro* maturation and parthenogenetic activation was replicated eight times with average 60 oocytes per replicate. In each step pools of oocytes (n = 30 from three different replicates) were randomly selected to monitor the

oocyte and/or embryo development at a specific designed stage.

Immunofluorescence detection of α-tubulin in oocytes and presumptive zygotes

Injected oocytes 18 h after fusion and oocytes 6 h post-activation were fixed in 4% paraformaldehyde (w/v) in phosphate buffered saline (PBS), pH 7.4 for 30 min at room temperature. After washing in PBS, oocytes were permeabilized by incubation in PBS containing 0.1% Triton-X100 (v/v) and then were blocked with 1% goat serum (v/v; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature then overnight at 4°C. Primary antibodies directed against alpha-Tubulin were used, mouse monoclonal [4G1] to alpha Tubulin (Abcam, Dawinbio Inc., Gyeonggi-do, Seoul, Korea) with dilution 1:100, was prepared in 1% goat serum. Oocytes were incubated in primary antibody solution for 2 h at room temperature, washed in PBS three times, then incubated with secondary antibodies (Cy3-conjugated goat anti-mouse IgG; Jackson ImmunoResearch laboratory Inc., West Grove, PA, USA) diluted 1:200 in PBS for 1 h at room temperature then washed in PBS three times. Nuclei were counterstained with Vectashield mounting medium containing 4′,6′-diamidino-2-phenylindole (DAPI; Vector Laboratories).

Nuclear staining and chromosome analysis

For observing the nuclear materials, injected oocytes and presumptive zygotes were stained by Hoechst 33343 (2 μmol/L in TALP) for 10 min, mounted on glass slides in a drop of glycerol and gently flattened with a cover glass. Nuclei were observed with a fluorescence microscope using a 346-nm excitation filter (Nikon TE2000, Tokyo, Japan). Cytogenetic analysis of the injected oocytes and the resulted blastocysts was performed according to Tarkowski (1966) and McGaughey & Polge (1971), respectively, with modification. Briefly, colcemide (0.2 mg/mL; Gibco, Grand Island, NY, USA) was added to the PZM5 medium and embryos were cultured for an additional 4 h. Oocytes and blastocysts (n = 10 each) were hypotonized with sodium citrate (0.8% [w/v]) for 3 min, followed by KCl (75 mmol/L) treatment for 2 min. Oocytes and blastocysts were fixed overnight in methanol–acetic acid (3:1), allocated individually onto clean slides and spread with a few drops of methanol–acetic-acid (1:1). The slides were then air-dried, stained with 5% (v/v) Giemsa stain (Gibco) for 5 min, rinsed with distilled water and air dried. The chromatids/chromosomes numbers were examined under oil immersion lens.

RNA extraction, complementary DNA (cDNA) synthesis and PCR

Total RNA was extracted and eluted from individual RFP-expressing blastocysts (n = 8) using the easy-spin (DNA-free). Total RNA Extraction Kit (iNtRON Biotechnology, Inc., KyungGi-Do, Korea) according to the manufacturer's instructions. RNA purity was evaluated through NanoDrop 2000 (Thermo Fisher Sci., Waltham, MA, USA) by estimating the ratios of absorbance at 230, 260 and 280 nm; values of A260/A280 of ≥2.0 and A260/A230 > 2.0 were accepted and used for reverse transcription (RT). Pulsed RT to increase RT efficiency was used as following; 40 cycles of 16°C for 2 min, 42°C for 1 min and 50°C for 1 s, followed by a final inactivation at 85°C for 5 min. Individual RT reactions were performed using 100 ng of total RNA, random hexamer and superscript III reverse transcriptase (Invitrogen) in a 20-μL reaction volume according to the manufacturer instructions.

One microgram cDNA was subjected to polymerase chain reaction (PCR) using Maxime PCR PreMix kit-*i*-starTaq (Intron Biotech., Seoul, Korea). The PCR amplification was carried out for one cycle of denaturation at 95°C for 5 min and subsequent 40 cycles with denaturation at 95°C, annealing for 30 s, extension at 72°C for 45 s and final extension at 72°C for 5 min. Ten microLitre of PCR products were fractionated on 1% agarose gel (Intron Biotech.) and stained with RedSafe (Intron Biotech.). In all assays, cDNA template negative and reactions without RT resulted in negative amplification. Specificity of PCR products was confirmed by direct sequencing (Macrogen, Seoul, Korea). The identity of each product was confirmed by sequence homology analysis using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) GenBank (http://blast.ncbi.nlm.nih.gov/) as we described previously (Saadeldin *et al.* 2014). Primer sequences, annealing temperatures and approximate size of the amplified fragments are listed in Table S1.

Statistical analysis

The ratios of embryo cleavage and blastocyst formation were evaluated using Pearson's χ^2 test. Statistical significance was considered when the P-value was <0.05.

Results

Fusion and division of the transferred somatic cells during oocyte maturation

Immature cumulus-free oocytes were juxtaposed with RFP-expressing cells and subjected to an electrical

pulse for fusion. After 1 h, the oocytes were observed under stereomicroscope and only fused couplets were used for IVM. After 18 h, a random sample of oocytes (n = 30, three replicates) was used for immunofluoresecnce staining against α-tubulin. Eighteen fused couplets (60%, Table S2) showed two spindles formation surrounding chromatin materials either at metaphase I (Fig. 1B) or were found to contain a dividing nucleus at anaphase I stage along with the presence of somatic nuclei surrounded with spindle formation (Fig.1C). However, nine oocytes (30%, Table S2) were found to contain dividing oocyte nucleus with abnormal or without spindle formation for the somatic nucleus (Fig. S2 A,B, respectively). After 44 h of IVM, 55.3% of oocytes showed presence of dividing nuclei (Fig. 2A, B, Table S3) and formation of two polar bodies (Fig. 2C) one of them showed RFP expression nearby the 1st polar body (Fig. S3 A,B). Chromosomal spread showed that 60% of the examined oocytes showed nucleus containing 19 paired chromatids beside a condensed nuclear materials which indicate the formation of polar bodies (Fig. 2D and Table S3), when compared to control non-injected oocytes (Fig. S2 C). These results suggest that somatic cell nuclei were obliged to undergo meiosis I along with the normal behavior of oocyte at 1st meiosis.

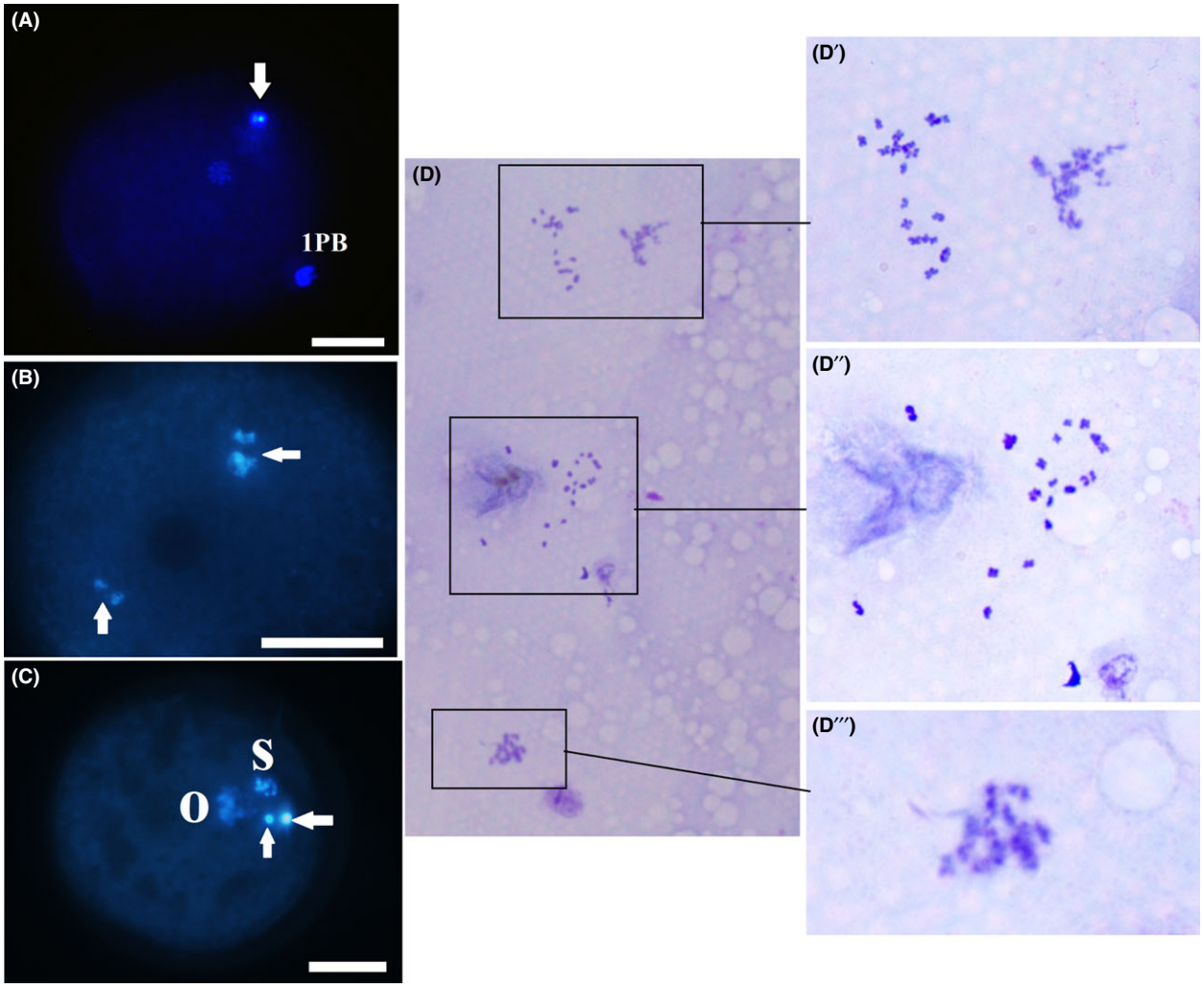

Fig. 2. Representative images of *in vitro* mature oocytes after fusion with somatic cells at premature stage. (A) Formation of 1st polar body (1PB); however, the divided nuclei of somatic cell is unable to separate completely (arrow). (B) Both somatic and oocyte nuclei are dividing at anaphase I stage (arrows). (C) Successful separation of somatic (s) and oocyte (o) nuclei and formation of two polar bodies (arrows). Scale bars = 20 μm. (D) chromosome smear showing two chromosomes set, 19 double clear chromatids each, indicating the somatic and oocytes chromosomes and two groups of condensed chromosomes indicating the two polar bodies, D′, D″ and D‴ are magnified parts of D.

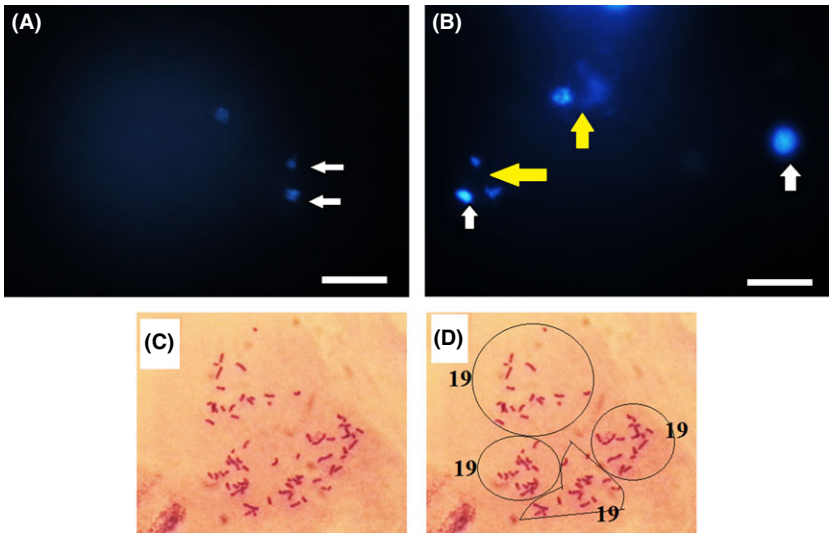

Fig. 3. Representative images of injected mature oocytes 6 h after electric activation. (A) The non-injected control oocyte showing a nucleus with two polar bodies (white arrows). (B) Division of somatic and oocyte nuclei (yellow arrows) and presence of the remained 1st polar bodies (white arrows). Scale bar = 20 μm. (C) chromosomes smear showed four groups, 19 chromatids or monads each.

Mature oocyte activation, haploidizationand pronuclear formation

Mature oocytes with two polar bodies were electrically activated and cultured for subsequent development. After 4–6 h, a random sample (n = 30) of presumptive zygotes was stained with Hoechst 33342. The oocytes showed presence of two divisions beside the first polar bodies (Fig. 3B) in 36.7% of examined oocytes when compared with the non-injected parthenogenetically activated mature oocytes (Fig. 3A and Table S4). Additionally, chromosomes smear showed that 46% of examined embryos contain four groups of 19 chromatids (monads) each (Fig. 3C,D and Table S4), which indicate the hapoidization or reduction division for both oocyte and injected somatic cell nuclei. After 12 h, activated oocytes were examined under UV rays to observe the 2nd polar bodies; some embryos protruded four polar bodies two of them expressed RFP suggesting the successful completion of 2nd meiosis for both oocyte and the fused somatic cells (Fig. S4 A, B).

Embryo development and blastocyst formation

Cleavage, morula and blastocysts. Cleavage was observed on day 2 of IVC. The cleaved embryos and embryos beyond the two-cell stages, including blastocysts, were examined for RFP expression through brief exposure to UV rays and were counted for RFP-expressing ones (Fig. 4). The rates of RFP-expressing two-cell embryos and blastocysts were 23.2% and 6.9%, respectively, (Table 1). Negative RFP expression in control parthenogenetic embryos (>two-cell stage embryos and blastocysts) is shown in Figure S5 A,B, respectively.

PCR and cytogenetic analysis of the resulted blastocysts

Polymerase chain reaction analysis showed expression of RFP mRNA in all red fluorescence-expressing blastocyst (n = 8). Expression of Xist mRNA was found in six blastocysts and expression of Sry mRNA was found in only one blastocyst (Fig. 5A). Among 11 blastocysts-expressing RFP-examined for chromosomal analysis; two blastocysts (18.18%, Fig. 5B) was found to be diploid (19 double-paired chromosomes) and the other nine blastocysts (81.82%) were found to be aneuploid (more than 38 chromosomes with abnormal morphology) (Fig. 5C–E).

Discussion

In the current approach, immature oocytes were fused with RFP-expressing somatic cells, matured *in vitro*, electrically activated and subsequently cultured; we observed RFP expression in the resulted embryos (23.2% >two-cell and 6.9% blastocysts).

In preliminary experiments, we injected RFP somatic cells into mature oocytes perivitelline space (n = 50 extruded 1st polar body) and then were electrically fused and activated as mentioned above, but we failed to get transgenic embryos. This result coincides with Fulka *et al.,* who found that somatic cell nuclei were unable to precede meiosis when introduced into an oocyte meiotic cytoplasm (Fulka *et al.* 2002); however, it contradicts with Fujii & Funahashi (2008) and Lacham-Kaplan *et al.* (2001) who showed that ovulated metaphase II oocytes injected with membrane-free somatic nuclei can form pronuclei and cleave following activation. Specifically, the experimental conditions of the latter two studies are different with ours because

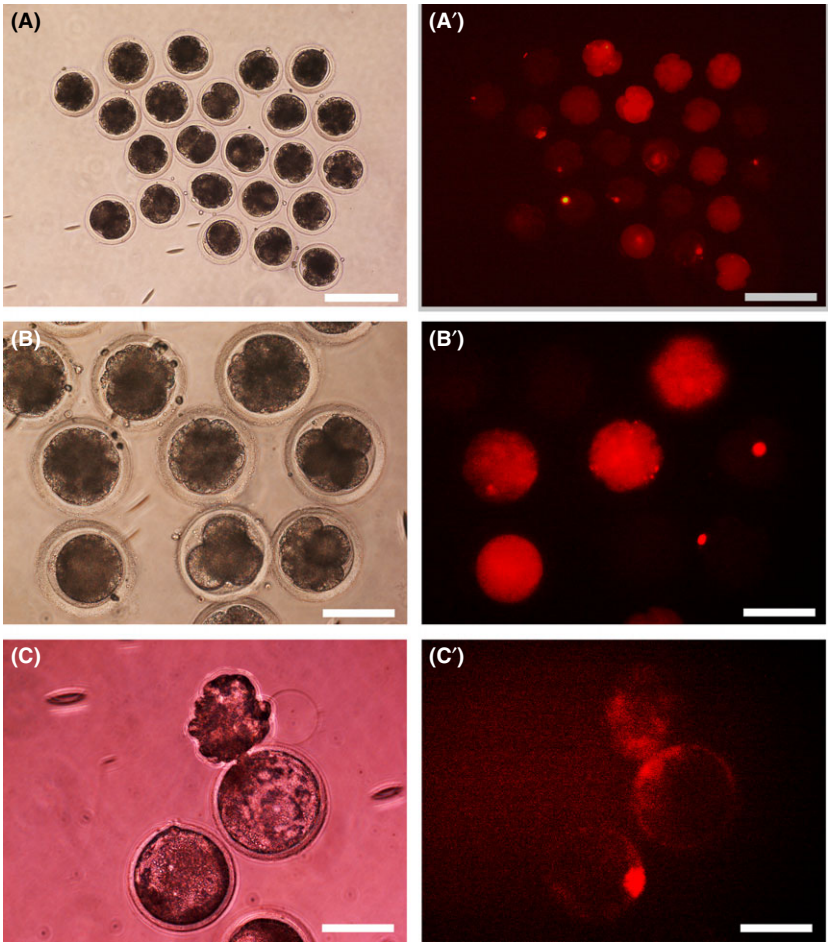

Fig. 4. Representative images of red fluorescent protein (RFP) expression in the resultant *in vitro* cultured embryos after electric activation of injected oocytes. (A) Two to four cell stages embryos scale bar = 200 μm. (B) >4-cell stage embryos. (C) Blastocyst stage. (A′), (B′) and (C′) represent images after UV rays exposure. Scale bar = 100 μm.

Table 1. Development of porcine immature oocytes after injection and fusion with red fluorescent protein (RFP)- expressing somatic cells, *in vitro* maturation and electric activation

	Control† DOs	Injected only	Injected and co-cultured‡
Number	125	125	275
Two-cell stage	55 (43.52%)[a] RFP−	0 (0%)[b]	64 (23.2%)[c] RFP+
Blastocysts	20 (16%)[a] RFP−	0 (0%)[b]	19 (6.9%)[c] RFP+

†Control means parthenogenetically activated without somatic cell injection.
‡Coculture of injected oocytes with cumulus-oocyte complexes and ZP-free parthenogenetically activated embryos.
Values in the same raw carrying different superscripts are significantly different.
DOs, denuded oocytes; −, negative expression; +, positive expression.

they injected somatic nuclei into the oocytes ooplasm after breaking both somatic cell membrane and the oolema; however, we used electric current for the whole cell fusion that might activate the meiosis II of oocyte before division of the somatic cells and caused asynchronous nuclear division.

From Table 1, it is apparent that the process of oocyte injection and fusion with the RFP- expressing cells has critically affected the developmental competence of the resulted embryos (two-cell stage was 0% vs 43.52% and blastocyst was 0% vs 16%, respectively, $P < 0.001$). However, it showed 6.9% of RFP-expressing blastocyst when co-cultured with COCs and ZF-parthenogenetically activated embryos, which indicates a problem in reprogramming of the fused foreign chromosomes. Several studies unraveled the important role of cumulus cells in supporting oocyte maturation and the embryonic development to the blastocyst stage (Maedomari *et al.* 2006; Zhang *et al.* 2010). Moreover, our recent results

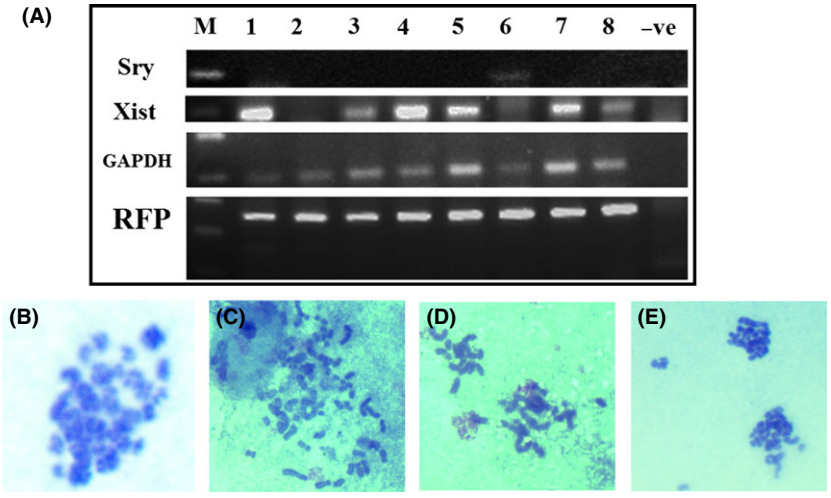

Fig. 5. Analysis of red fluorescent protein (RFP)-expressing blastocysts. (A) Photomicrograph of gel electrophoresis of polymerase chain reaction (PCR) products showing the expression of Sry, Xisr, GAPDH Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and RFP mRNAs in eight blastocysts (n = 1–8), M is DNA base pair marker, -ve is PCR reaction without cDNA template. B–E represent cytogenetic analysis of the resulting blastocysts; (B) Diploid. (C–E) Aneuploid with different chromosomes morphology.

showed improvement of low quality embryos when co-cultured with zona-free parthenogenetic embryos in microdrops, because the later secretes essential reprogramming factors, such as Oct4, Sox2, c-Myc and Klf4 in the culture medium (Saadeldin *et al.* 2014).

The results showed that injected nuclei were able to undergo: meiosis I; meiosis II with haploidization; pronuclear formation and integration of genetic materials into the zygote genome as well as its functioning represented as RFP expression throughout the preimplantation embryo developmental stages (two-cell stage till the blastocyst).

Among eight blastocysts, Xist mRNA expression was found in six, while expression of Sry mRNA was observed in only one blastocyst. A result that may suggest difference in behaviors of X and Y chromosomes in the oocyte meiosis process (Villemure *et al.* 2007; Alton *et al.* 2008); i.e. Y chromosome may be segregated with the 1st or 2nd polar bodies; however, further study is needed in this regard. Expression of Sry mRNA might indicate that Y-chromosome paired with maternal X-chromosome and proceeded till embryo formation (Obata *et al.* 2008). One blastocyst was neither expressed Xist nor Sry, which might be caused by the aneupolidy and/or impairment in gene expression in the resulting embryos (Lyle *et al.* 2004; Lightfoot *et al.* 2006).

Among blastocysts that were used for chromosomal analysis, 81.82% showed aneuploidy which coincides with (Fujii & Funahashi 2008) who showed incompatibility among the donor and maternal chromosomes; however, 18.18% showed diploid number of chromosomes which suggests a possible compatibility among the donor and maternal chromosomes after somatic cell fusion. The approach and results is illustrated in Figure S6.

If further improved, this approach would allow the production of embryos for a human couple where the male partner is sterile. This approach might be also used as a shortcut tool for genome editing and/or transgenesis in males and its progeny through: (i) correcting target gene in Y-linked diseased male partner, or (ii) gene addition in gene-deficient male partner. Moreover, this approach provides a paradigm for understanding the molecular behavior of meiosis I and the ooplasmic kinetics during the interaction with foreign chromosomes, especially Y-chromosome**.**

In conclusion, we propose "prematuration somatic cell fusion" as an approach to generate embryos using somatic cells instead of sperms; however, it needs further improvement and precise chromosomal analysis of the resulting embryos before transfer to the surrogate mothers**.**

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Vector used for transfection and generating red fluorescent protein (RFP)-expressing cells.

Fig. S2. Immunofluorescence staining of α-tubulin showing oocytes having dividing nucleus with abnormal (A′, arrow) or without (B) spindle formation for the injected somatic nucleus.

Fig. S3. (A,B). Injected oocytes showed cytoplasmic part with red fluorescent protein (RFP) expression after UV exposure (B) nearby the 1st polar bodies (A, white arrows) after 44 h of *in vitro* maturation (IVM). Scale bar = 200 μm. C,C-. Control non-injected oocytes showing 19 chromsomes (magnified in C-) with the 1st polar body (PB).

Fig. S4. Embryos protruded four polar bodies (A) two of them expressing red fluorescent protein (RFP) (B) suggesting the successful completion of 2nd meiosis for both oocyte and injected cells.

Fig. S5. Representative images of control non-injected parthenogenetically activated oocytes showing no red

fluorescent protein (RFP) expression either in cleaved embryos (A,A′) or in blastocyst stage (B,B-). Scale bar = 200 μm and 100 μm, respectively.

Fig. S6. Illustration for the prematuration somatic cell fusion approach. Injected and fused somatic cell divides and doubles chromosomes numbers (2N/4C).

Table S1. Primer sequences and product size used for RT–PCR.

Table S2. Spindle formation (α-tubulin) in premature oocytes injected and fused with somatic cells after 18 h from *in vitro* maturation (IVM).

Table S3. Polar body formation in premature oocytes injected and fused with somatic cells after 44 h from *in vitro* maturation (IVM).

Table S4. Six hours post-activation of premature oocytes injected and fused with somatic cells after 44 h of *in vitro* maturation (IVM).