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Original Article

Blastomeres aggregation as an efficient alternative for trophoblast culture from porcine parthenogenetic embryos

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Zona pellucida free (ZPF) oocytes were cultured after electrical activation to allow blastomeres aggregation and compared to ZP intact (ZPI) oocytes. In feeder-dependent conditions, the trophoblast attachment and primary outgrowths were significantly higher in ZPF than in ZPI groups. In feeder-free conditions, trophoblast attachment and typical morphological trophoblast primary outgrowths were observed in ZPF group. The primary colonies derived from the ZPF embryos in both culture conditions were able to establish secondary and tertiary colonies and showed mRNA expression of *CDX2*, *TEAD4* and *KRT8* as trophoblast markers, while outgrowths from the ZPI embryos could not grow beyond primary colonies.

Key words: blastomeres aggregation, parthenogenesis, pig, trophoblast cells.

Introduction

In mammals, the trophoblast lineage of the embryo is specified before implantation. It is restricted to become the fetal portion of the placenta. Recent attempts failed to obtain trophoblast passages from *in vitro* produced porcine embryos particularly parthenogenetic (PA) embryos (Roberts & Fisher 2011; Vackova *et al.* 2011) because of the big difference in cell numbers when compared to *in vivo* derived embryos and the hatching problem caused by zona hardening and thickness (Papaioannou & Ebert 1988; Brevini *et al.* 2010). Recently, we reported an assisted hatching method to overcome the problems with porcine parthenogenesis to improve the trophoblast culture (Saadeldin *et al.* 2014b); however, this method requires a micromanipulator which is not accessible in many laboratories.

Several trials describing embryonic stem cell (ESC)-like cultures showed difficulties in the pig that might be due to the lack of specific markers exclusive to ESCs and trophoblasts due to the unique features of the preimplantation development in this species (Hall & Hyttel 2014).

Haploid cells provide a valuable *in vitro* model to study the genomic imprinting and understanding the differentiation process in monoparental cells (Cibelli *et al.* 2006; Brevini & Gandolfi 2008; Hall 2008; Shan *et al.* 2012). Organisms with a single copy of their genome provide a basis for genetic analyses where any recessive mutation of essential genes will show a clear phenotype due to the absence of a second gene copy (Carette *et al.* 2009).

Here, we show that it is possible to generate porcine trophoblast outgrowths from parthenogenetic pig blastocysts derived from blastomeres aggregation. This would act as an alternative method to produce PA embryos with advantageous characters including increased cell count and capability of attachment and derivation of trophoblast cells either on feeder-dependent or -independent culture conditions.

Methods

Chemicals

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

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Ovaries and cumulus-oocyte complexes recovery and in vitro *maturation*

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 *in vitro* maturation on the basis of morphological features, i.e. a compact multi-layered cumulus mass and a dark, evenly granulated cytoplasm. The COCs were cultured in four-well dishes (50 COCs per well; Falcon, Becton Dickinson, Plymouth, UK) in basic maturation medium, TCM-199 supplemented with 10 ng/mL epidermal growth factor (EGF), 0.57 mM cysteine, 0.91 mM sodium pyruvate, 5 μg/mL insulin, 1 μg/mL Follicle stimulating hormone (FSH) (Antrin, Teikoku, Japan) and 1% (v/v) Pen-Strep (Invitrogen) at 39°C in a humidified atmosphere of 5% CO_2 for 44 h (two stages, 22 h each with hormonal removal in the second stage). After 44 h, oocytes and expanded cumulus cells were separated by pipetting with 0.1% hyaluronidase in Tyrode's albumin lactate pyruvate (TALP).

Zona pellucida removal, parthenogenetic activation and in vitro *culture of matured oocytes*

Cumulus-free denuded oocytes were divided into two groups. The first group was treated with pronase 0.3% (w/v in phosphate-buffered saline [PBS]) for 1.5 min or until zona pellucida (ZP) digestion and then was quickly washed in TALP three times; zona pellucida free (ZPF) oocytes. The other group was kept without pronase treatment; zona pellucida intact (ZPI) oocytes. ZPF and ZPI oocytes (n = 500 of each) were sequentially equilibrated in a gradient concentration (0%, 33%, 66% and 100%) of mannitol solution (0.25 M) in a four-well dish. Technically, ZPF oocytes are lighter than ZPI ones and take a long time (1–2 min) to settle down and equilibrate in mannitol solution. The oocytes were then transferred into a two electrodes mannitol chamber connected with a BTX electrocell manipulator 2001 (BTX, San Diego, CA, USA) and activated by a single pulse of 1.5 kV/cm for 100 μs and were kept for 3 min in the activation medium according to our previous report (Saadeldin *et al.* 2014a). 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. Embryo density was fixed in both groups 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, 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.

Total and differential blastocyst cell count

We followed the method described by (Thouas *et al.* 2001) with minor modifications. In brief, three to five hatched blastocysts from each experimental group were first incubated in 500 μL of Solution 1 (BSA-free Hepes buffered TCM-199 medium supplemented with 1% Triton X-100 and 100 μg/mL propidium iodide) for 25 s. Blastocysts were then immediately transferred into 500 μL of solution 2 (fixative solution of 100% ethanol with 25 μg/mL Hoechst 33342 for 10 min at room temperature. Stained blastocysts were mounted on a glass slide in a drop of glycerol, gently flattened with a cover glass and visualized for cell counting with a fluorescence microscope using a 346-nm excitation filter. Trophoblast cells appear as pink fluorescence while inner cell mass cells appear as blue fluorescent. Digital photographs were taken for total and differential cell counting using ImageJ 1.42q software.

Trophoblast culture in feeder-dependent and feeder-free conditions

Inner cell mass (ICM) cells were microsurgically removed using sterilized blade under stereomicroscope and the remaining trophoblast portion was cultured for 6–12 h to gain the vesicular shape. For feeder-dependent culture conditions, trophoblast vesicles (TVs) of ZPF and ZPI groups were plated into 4-well tissue culture dishes (Nunc, Thermo Scientific, Roskilde, Denmark); 1–2 TVs/well. The dishes were coated with 0.1% gelatin and containing feeder layers of mouse embryonic fibroblasts (MEF) mitotically inactivated with mitomycin C (Sigma-Aldrich). In feeder-free conditions, trophoblast vesicles were placed on the surface of basement membrane matrix (known as Matrigel) freshly coated culture dishes; 1–2 TVs/well. Briefly, previously cooled (−20°C) 4-well culture plates were covered with 120 μL Matrigel (BD Biosciences, San Jose, CA, USA) prepared according to the manufacturer's instructions. The culture medium was a mixture of DMEM/F-12 (HAM) 1:1 supplemented with 10% fetal bovine serum (FBS), 0.1 mM, β-mercaptoethanol, 1% nonessential amino acids (Invitrogen), and 1% Penicillin/Steptomycin (Invitrogen). Fresh medium was added to the primary cultures every 3–4 days.

Secondary and subsequent cultures were done by removing the cells monolayer from the tissue culture plate surface followed by mechanical dissociation and chopping of the primary colonies and subculture of the small chops on fresh culture plates. Incubation performed at 38.5°C in a humidified atmosphere of 5% CO_2 air.

RNA extraction, complementary DNA (cDNA) synthesis and PCR

Total RNAs were extracted and eluted from TVs ($n = 3$) and cultured colonies ($n = 3$ of each group) using the easy-spin (DNA-free) Total RNA Extraction Kit (iNtRON Biotechnology, KyungGi-Do, Korea) according to the manufacturer's instructions. RNA purity was evaluated through NanoDrop 2000 (Thermo Fisher, 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 are accepted and used for reverse transcription (RT). Pulsed RT was used to increase RT efficiency 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 μL 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.* 2014a). Semi-quantification of intensity of RT-PCR signals was carried out by densitometric scanning using ImageJ 1.42q software (NIH, USA) and values of the specific targets were normalized to those of the internal control (*GAPDH*) to express arbitrary units of relative expression. Primer sequences, annealing temperatures and approximate size of the amplified fragments are listed in Table S1.

Chromosome analysis of the resulted blastocysts

Cytogenetic analysis was performed according to McGaughey and Polge (1971) 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. Blastocysts ($n = 10$) were incubated in hypotonic sodium citrate (0.8% (w/v)) for 3 min, followed by KCl (75 mM) treatment for 2 min. Blastocysts were fixed overnight in methanol–acetic acid (3:1), allocated individually onto clean slides and cells were spread with a few drops of methanol–acetic-acid (1:1). The slide was then air-dried, stained with 5% (v/v) Giemsa (Gibco) for 5 min, rinsed in distilled water, air dried. The chromatids/chromosomes numbers were examined under oil immersion lens.

Statistical analysis

The ratios of blastocysts formation, hatching and the resulted trophoblast outgrowths in both groups were evaluated using Pearson's χ^2 test. The means ± SEM of cell counts in both groups were compared using Student's *t*-test. Statistical significance was considered when *P*-value was less than 0.05.

Results and discussion

Unlike the conventional parthenogenesis using zona intact oocytes (ZPI), we removed the ZP by pronase and allowed the oocytes to cleave in microdrops culture system. Several reports used the well-of-the-well (WOW) system to culture 1–3 Zona pellucida free (ZPF) embryos (Vajta *et al.* 2008; Shan *et al.* 2012); however, we found using more embryos with small volume of medium in mircodrops was easier for manipulation and gave satisfactory results. In some trials we found that embryos began to cleave and form blastomeres as soon as culture conditions started (Fig. 1A shows the cleavage after 6 h of culture). Blastomeres were found to clump to form a solid mass-like aggregation (Fig. 1B). The reason behind this aggregation and compaction is unknown and might be due to the formation of cytonemes or filopodia by the blastomeres as shown by Fierro-González *et al.* (2013); however, further study is needed. Interestingly, fluid-filled vesicles or blastoceles started to protrude from the aggregation by the end of day-4 (Fig. 1C) and largely expanded by day-6 and 7. To obtain the blastocysts, gentle pipetting should be performed to remove the cell debris and to separate the blastocyst from the surrounding aggregates.

The rate of blastocyst formation was significantly lower in ZPF (105/500, 21%) than ZPI (170/500, 34%) ($P = 0.001$); however, the problem of hatching makes

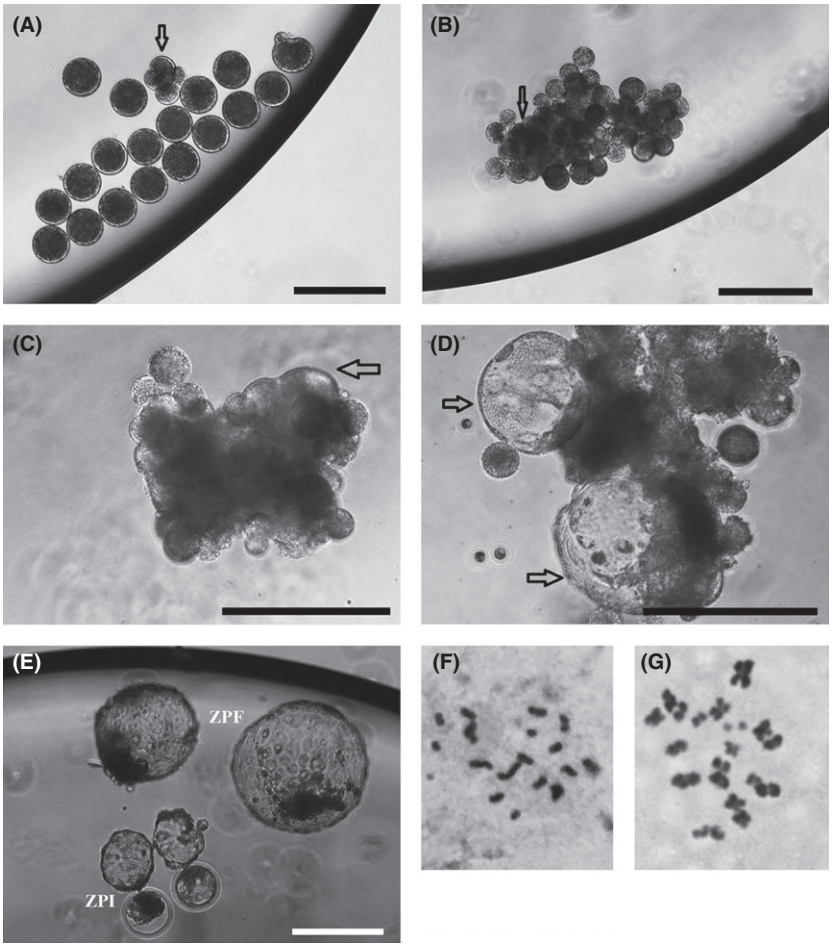

Fig. 1. Development of parthenogenetic embryos after blastomeres aggregation. Zona pellucida (ZP) of mature oocytes was digested by pronase and was then electrically activated. A total of 15–20 activated zona pellucida free (ZPF) oocytes were cultured in microdrops of PZM5. (A) Some embryos start to cleave and form blastomeres as early as 6 h post-activation (arrow). (B) On day-2 the blastomeres start to form a solid mass-like aggregation (arrow). (C) Blastocele formation starts to protrude from the aggregation by the end of day-4 (arrow). (D) A vast expansion of the resultant trophoblast vesicles (arrows) start to protrude on day-6 with the attachment of inner cell mass (ICM) cells to the aggregation side. (E) ZPF blastocysts were gently pipetted to remove the extra aggregated and dead cells and clearly show the ICM and TE cells and compared with the ZPI blastocyst. (F, G) Cytogenetic analysis of ZPF blastocysts showing 19 single and paired chromatids, respectively. Scale bar = 200 μm.

the ZPF significantly advantageous over the ZPI method because the hatching % in ZPI was 15.8% (n = 27/170) if compared to 100% in ZPF group (P = 0.00). We recently overcame the problem of hatching in PA embryos through assisted hatching using mature oocyte zona puncture (Saadeldin *et al.* 2014b). Moreover, ZPF blastocysts showed significant increase in total and differential cell number (approximately threefold) than those of ZPI ones. Total cell count was 49.2 ± 13.2 in ZPI while it was 172.8 ± 48.4 for ZPF (P = 0.019). Inner cell mass cells counts were 13.4 ± 3.1 and 34.6 ± 2.6 (P = 0.019) in ZPI and ZPF groups, respectively. Moreover, trophectoderm cell counts were 35.8 ± 10.5 and 138 ± 34.2 (P = 0.035) in ZPI and ZPF groups, respectively.

We examined the chromosomes number of the resulted blastocysts (n = 10) through cytogenetic analysis and were found to be haploid; 19 single chromatids in the G1 stage of the interphase (Fig. 1F) and 19 paired chromatids held together by the centromere at the metaphase (Fig. 1G) of the cell cycle.

The resultant blastocysts were then used to culture embryo-derived trophoblast cells using feeder layers or basement membrane matrix (Matrigel). We isolated trophoblast vesicles (TVs) through microsurgery. Both groups showed variable behaviors and capabilities of attachment to the culture surface, to form outgrowths, and to continuously proliferate with maintaining the apparent trophoblast morphology. In ZPI group, 18.1% (n = 2/11) of TVs were able to attach and grow on

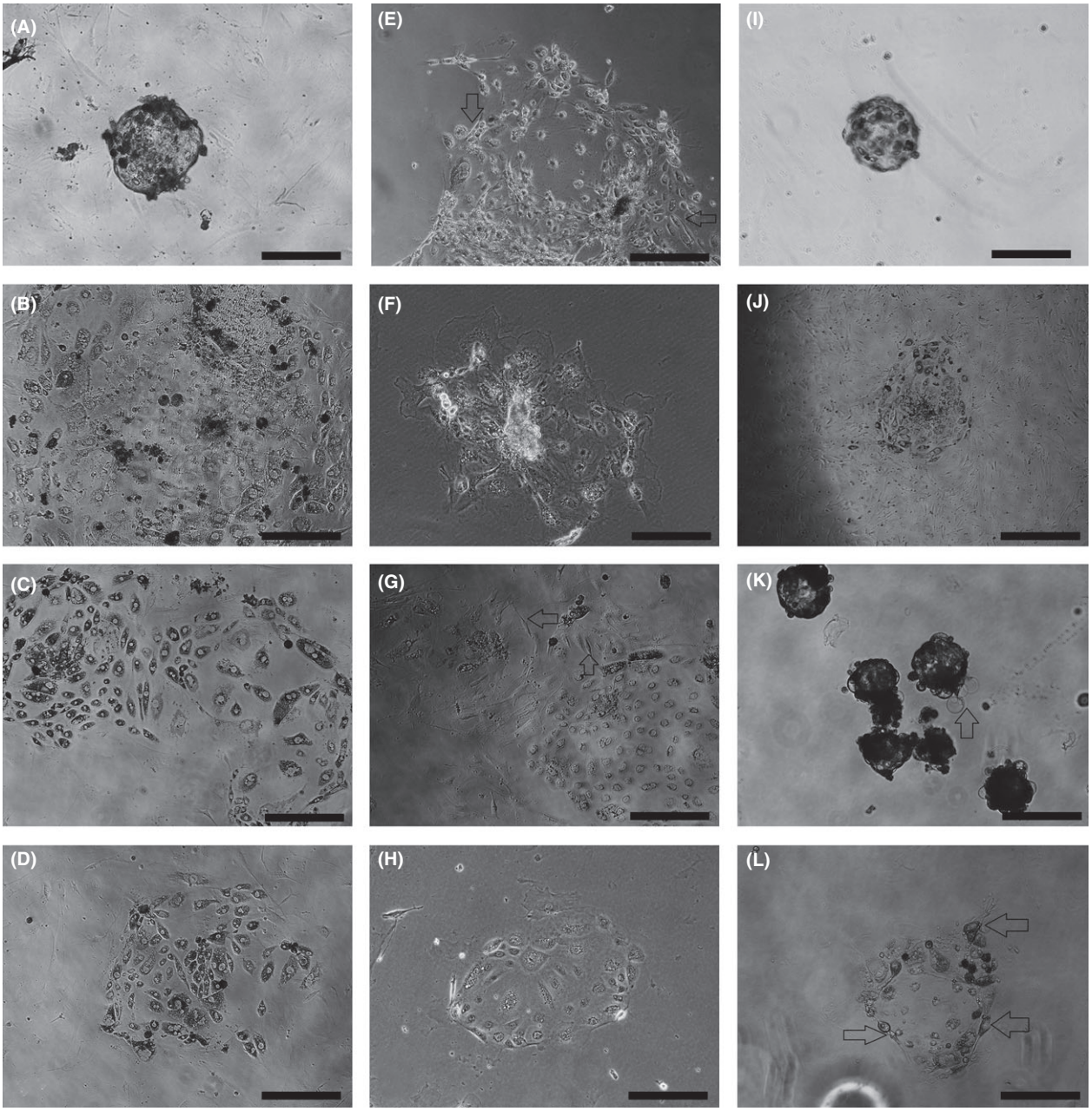

Fig. 2. Maintenance of trophoblast cells derived from blastomeres aggregation on feeder cells and Matrigel matrix. Trophoblast vesicles of zona pellucida free (ZPF) (A) and ZPI (I) groups were obtained by microsurgery and then were placed on culture dishes. (A–D) ZPF group cultured on feeder layer. (B) Primary cultured ZPF trophoblast vesicles show presence of cuboidal cells of varied sizes. (C) Typical morphology of trophoblast cells in the second passage. (D) Typical trophoblast morphology maintained at the third passage. (E, F) ZPF group cultured on Matrigel. (E) Primary cultured ZPF trophoblast vesicles shows presence of cuboidal cells with some elongated spindle shaped cells (arrows). (F) The cells are unable to divide and differentiate to abnormal morphology. (G, H) ZPF group first cultured on feeder layer then were passaged over Matrigel. (G) Presence of typical trophoblast morphology with appearance of elongated and spindle shaped cells (arrows). (H) Typical trophoblast morphology maintained at the third passage with varied cell size. (I, J) ZPI TVs growth on feeder layer. (J) ZPI group can form primary outgrowth with typical trophoblast morphology but unable to grow beyond the first passage. (K, L) ZPI group cultured on Matrigel. (K) Trophoblast vesicles unable to attach to Matrigel matrix and undergo apoptosis as indicated by presence of cystic embryoid bodies (arrows). (L) The attached vesicles form abnormal morphological cells with spindle poles. Scale bar = 200 μm.

feeder layer; however, they could not be passaged further (Fig. 2I,J). Additionally, 9.1% (n = 1/11) of TVs were able to attach to the Matrigel matrix but showed different morphological characters (Fig. 2K,L). The failure of attachment to Matrigel matrix causes the embryonic cells to form cystic embryoid bodies (Fig. 2K arrows) which indicate cellular apoptosis (Brown *et al.* 2006; Saadeldin *et al.* 2012). On the other hand, 86.8% (n = 13/15) of ZPF TVs were able to attach and grow on feeder layer. In the primary passage, there were some mixed cells of different morphologies, which might be related to the naïve characters of porcine embryonic trophoblasts (Hall & Hyttel 2014). Meanwhile, the typical morphology of the trophoblast (large and cuboidal cells with granular cytoplasm) was observed in feeder-dependent conditions after the third passage (Fig. 2A–D). Even though 80% (n = 12/15) of ZPF TVs were able to attach to Matrigel, primary culture cells showed morphological changes; cells become thinner and show spindle morphology (Fig. 2E and Video S1) and after mechanical passage to a new fresh culture the cells lost the ability to grow and to maintain the trophoblast typical morphology (Fig. 2F). In another group (n = 15 TVs), ZPF TVs were cultured primarily on feeder layers then second passage was done using fresh Matrigel after mechanical chopping of the primary trophoblast colonies, but we found some cells morphologically changed to epithelial-type cells with elongated spindle shape instead of compact colonies together with the formation of typical morphology of trophoblast colonies (Fig. 2G). Typical trophoblast cells were picked mechanically and transferred to fresh Matrigel and formed a new colony with similar morphology to the trophoblast colonies (Fig. 2H). A previous study showed that trophoblast cell morphology can be affected by the culture surface (Stewart *et al.* 2004) and that is consistent with our finding.

Next, we analyzed the mRNA profile of pluripotency and trophoblast markers of the resultant colonies compared to their expression in the TVs (Fig. 3). We excluded the possibility of false positive results of using MEF mRNA in case of cultured colonies on feeder layer; results showed negative PCR reaction after using the same primers and PCR conditions for isolated total RNA and synthesized cDNA from MEF. In the current results, TVs showed expression of *Oct4* and *c-Myc* as pluripotency markers; however, mRNAs of *Sox2* and *Nanog* were not detected, which reflects the unique expression of the pluripotency markers in porcine embryos as recently described (Du Puy *et al.* 2011; Hall & Hyttel 2014). Trophoblast markers *CDX2*, *KRT8* and *TEAD4* were expressed in TVs in accordance with previous studies (Strumpf *et al.* 2005; Nishioka *et al.* 2008; Ezashi *et al.* 2011). Primary colonies on a feeder layer (Fig. 3-1F) expressed pluripotency markers including Nanog and the trophoblast markers in addition to mesenchymally-derived cells and cytoskeleton differentiation marker vimentin (*VIM*). However, primary colonies cultured on Matrigel (Fig. 3-1M) showed absence of Oct4, Sox2 and Nanog with reduction in the expression of trophoblast markers *CDX2*, *KRT8* and *TEAD4* (Fig. S1). In addition, the absence of *VIM* correlated with the morphological changes as viewed in Figure 2e. Interestingly, colonies of typical trophoblast morphology resulting from the third passage on feeders (Fig. 3-1F/3F) showed reduction in pluripotency markers *Oct4*, *c-Myc* and *Klf4* (Fig. S1) with absence of *Nanog* and

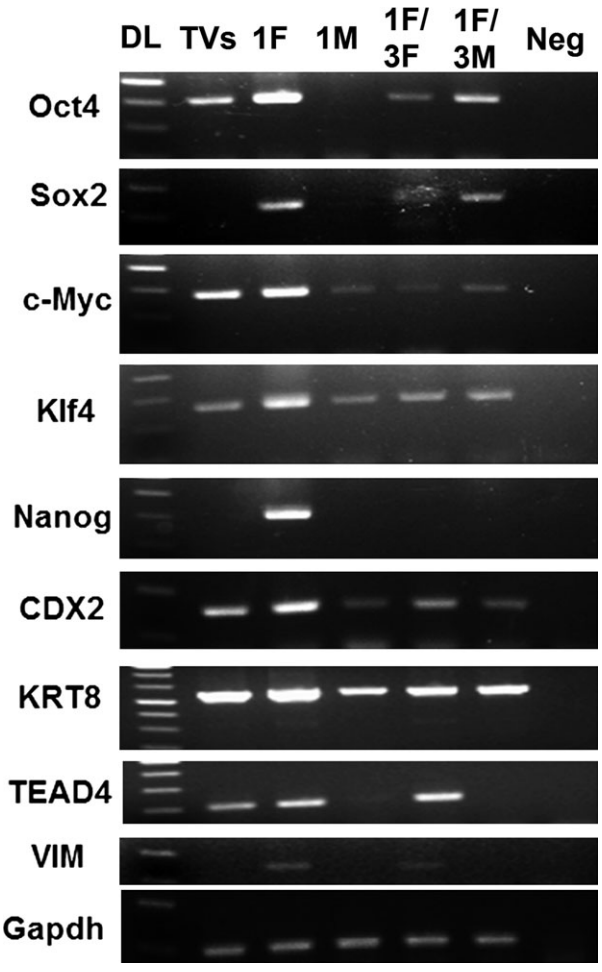

Fig. 3. Photomicrograph of gel electrophoresis of reverse transcription–polymerase chain reaction (RT–PCR) products showing expression of pluripotency mRNAs (*Oct4*, *Sox2*, *c-Myc*, *Klf4* and *Nanog*), trophoblast markers (*CDX2*, *KRT8* and *TEAD4*) and vimentin (*VIM*) in the ZPF trophoblast vesicles (TVs) and the resultant colonies. (1F) Primary culture on feeder layer. (1M) Primary culture on Matrigel. (1F/3F) Culture was maintained on feeder till the third passage. (1F/3M) Vesicles were primarily cultured on feeder layer then subsequent passages on Matrigel till the third passage; DL, DNA ladder; Neg, cDNA template was removed for negative reverse transcription.

maintenance of expression of trophoblast markers and *VIM*, reflecting that these colonies tend to be authentic trophoblast cells. The reason of *VIM* expression might be that porcine trophoblast-derived cells possess epithelial characteristics as well as have some mesenchymal characteristics. On the other hand, colonies of similar trophoblast morphology resulted from the third passage on Matrigel that was primarily cultured on feeders (Fig. 3-1F/3M) showed similar expression to 1F/3F colonies with absence of *TEAD4* and *VIM*, reflecting the morphological changes observed as mentioned above. *Nanog* expression in primary trophoblast cultured on feeders and its disappearance in the resultant trophoblast colonies may indicate the naïve characters of the freshly isolated trophoblasts.

From these results we can conclude that blastomeres aggregation provides an efficient alternative method to isolate and culture parthenogenetic trophoblast cells.

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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. Semi-quantitative analysis of RT-PCR gel electrophoresis images using pixel analysis.

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

Video S1. Time-lapse video showing culture of porcine primary trophoblast on Matrigel for 18 h.