Zygote

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Establishment of trophoblast cell line derived from buffalo (*Bubalus bubalis*) parthenogenetic embryo

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Research Article

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Summary

We have established trophoblast cell lines, from parthenogenesis-derived buffalo blastocysts. The buffalo trophoblast cells were cultured continuously over 200 days and 21 passages. These cells were observed by phase-contrast microscopy for their morphology and characterized by reverse transcriptase polymerase chain reaction and immunofluorescence against trophoblast-specific markers and cytoskeletal proteins. Trophoblast cells showed positive staining for CDX2, a marker of these cells at both blastocyst and cell line levels. Epithelial morphology of these cells was revealed by positive staining against cytokeratins and tubulin but not against vimentin and dolichos biflorus agglutinin. Gene expression profiles of many important placenta-specific genes were studied in the primary trophectoderm outgrowths, which were collected on days 0, 5, 9, 12 and 15 of culture and trophoblast cell line at passages 12–15. Therefore, the trophoblast cell line derived can potentially be used for *in vitro* studies on buffalo embryonic development.

Introduction

During early mammalian development, the trophoblast or trophectoderm (TE) cells are the first cells to differentiate in the embryo and contribute to the extraembryonic components of the placenta. While the inner cell mass (ICM) forms the embryo properly, the TE forms a significant portion of the placenta (Carlson, 1996). These cells are highly proliferative and invasive, making them a useful model for understanding placental growth disorders that occur after nuclear transfer technology. Trophoblast cell cultures or cell lines have been reported in domesticated species, such as pigs (Flechon *et al.*, 1995), goats (Miyazaki *et al.*, 2002) and cattle (Shimada *et al.*, 2001; Talbot *et al.*, 2000), and have been used for cell differentiation studies. The differentiation of TE is essential for implantation and placenta formation. This is due to their secretory properties, as they release key molecules such as interferon tau and placental lactogen (PL). Interferon tau, secreted at higher levels from mononucleate TE cells during implantation, is required for maternal recognition of pregnancy in ruminants. CDX2 is a marker of the TE and is responsible for TE lineage commitment. In the proliferating TE, CDX2 is expressed but disappears when these cells differentiate further into derivatives such as giant cells or spongiotrophoblasts in the mouse placenta (Simmons and Cross, 2005). Talbot *et al.* (2004) reported a bovine trophectoderm cell line derived from a parthenogenetic blastocyst. To date, there is no published report available on trophoblastic culture derived from buffalo parthenogenetic embryos. However, a trophoblastic cell line derived from buffalo *in vitro* fertilization (IVF) and a cloned embryo was also reported in our previous study (Mohapatra *et al.*, 2015). Establishing *in vitro* models of TE in buffalo can reveal the biology of implantation and placental development. Here, we present the isolation method, culture conditions and characteristics of TE cells derived from buffalo parthenogenetic embryo in an *in vitro* system which is the first report of its kind.

Materials and methods

All chemicals were obtained from Sigma Chemical, St. Louis, MO, USA, the media were purchased from GIBCO (USA) and disposable plasticware was purchased from Nunc (Denmark) unless otherwise mentioned. Fetal bovine serum (FBS) was obtained from HyClone (USA).

Ethics approval

As ovary samples were collected from a slaughterhouse (New Delhi, India), so there was no need of ethical approval.

Production of parthenogenetic embryos

Parthenogenetically derived embryos were produced as described earlier (Singh *et al.*, 2021). Briefly, *in vitro* matured Cumulus Oocyte Complexes were denuded by hyaluronidase (0.5 mg/ml) for 2 min to remove cumulus cells, following which, the zona was digested by pronase (2.0 mg/ml) treatment for 10 min. The zona-free oocytes were activated by treatment with 4 µM calcimycin A23187 for 5 min at 38.5°C followed by incubation with 2 mM 6-dimethyl aminopurine in T20 medium (T denotes HEPES-modified M-199 supplemented with 2.0 mM L-glutamine, 0.2 mM sodium pyruvate, 50 µg/ml gentamicin and 20% FBS) for 4 h in a CO_2 incubator at 38.5°C. The presumed parthenotes were then cultured in Research Vitro Cleave medium (K-RVCL-50, Cook® Australia, Queensland, Australia) supplemented with 1% fatty acid-free BSA in a 4-well dish (15–20 embryos per well), covered with mineral oil and kept undisturbed in a CO_2 incubator (5% CO_2 in air) for 8 days.

Isolation and culture of trophoblast cells

Mitomycin C-inactivated buffalo fetal fibroblast (BFF) feeder layers were prepared as described earlier (Sharma *et al.*, 2011). Day 8 parthenogenesis-derived blastocysts were seeded on the feeder layer as described earlier by Mohapatra *et al.* (2015). Briefly, the blastocysts were gently pressed on the feeder layer with the help of a 27 G hypodermic needle in 100 µl droplet of TE culture medium (DMEM + 10% FBS + 2 mM L-glutamine + 50 µg/ml gentamicin sulfate + 1% non-essential amino acids + ITS (I3146-5ML, Sigma, USA) and were cultured in a CO_2 incubator at 38.5°C. The primary TE cell colonies appeared within 2 weeks as a tight monolayer of cuboidal cells. The outgrowths were mechanically dissected using a microblade for the separation of these cells from the inner cell mass cells and were subcultured in a 1:2 split ratio, which was maintained for the first 2–4 passages. The cells were further subcultured every 10–12 days, by mechanical dissociation, on a fresh feeder layer in a split ratio of 1:4 till they remained alive in culture. The colony morphology was observed regularly under a Nikon phase-contrast microscope (Eclipse Ti, Nikon, Tokyo, Japan).

For the gene expression study of primary TE outgrowths, Day 8 parthenogenesis-derived blastocysts were seeded on MaxGel (E0282-1ML, Sigma, USA) coated dishes. For seeding of blastocysts on MaxGel Extra cellular Matrix (ECM)-coated dishes, 10 µl of MaxGel ECM/ml of DMEM was pipetted out in 48-well plates (400 µl/well), which were incubated for 2 h at 37°C in a CO_2 incubator. Then the medium was removed after 2 h, and fresh TE culture medium was added with seeding of blastocysts. The primary TE outgrowths were collected on days 0, 5, 9, 12 and 15 of culture. The relative transcript level of *IFN-tau*, *FGFR2*, *DNMT1*, *DNMT3a*, *CDX2*, *PAG2*, *ELF5*, *ETS2*, *GATA3* and *GATA2* was compared among different groups.

Characterization of TE cells

The morphology of TE cells was examined regularly under a phase-contrast microscope. The TE cells were characterized at regular intervals by examination of the expression of TE-specific markers by reverse transcriptase polymerase chain reaction (RT-PCR) and immunofluorescence. *OCT4*, *PAG2*, *IFN-tau* and *CDX2* were examined by RT-PCR. Primers were designed using the internet-based software PRIMER-3 (http://www-genome.wi.mit.edu/cgi-bin/prime/primer3-www.cgi). Total RNA was isolated from the TE cells using TRIzol (15596026, Invitrogen, USA) reagent according to the manufacturer's protocol. RNA integrity was checked by gel electrophoresis on 1.5% agarose, which showed two bands of 28S and 18S. DNase treatment was done to remove genomic contamination using a DNA-free kit (AM1906, Ambion, USA). The quality of RNA was checked by NanoQuant (Tecan, Salzburg, Austria). The RNA (1 µg) was reverse transcribed by RevertAid first strand cDNA synthesis kit (K1621, Thermo Scientific, Waltham, MA, USA), as per the manufacturer's protocol. The PCR reaction was performed in a thermal cycler (Bio-Rad, USA) using the following programme: initial denaturation at 95°C for 3 min, followed by 95°C for 30 s, annealing temperature for 30 s, 72°C for 30 s for 39 cycles and 72°C for 10 min in the last cycle. The annealing temperature and PCR conditions of the target genes are given in Supplementary Table 1.

Immunofluorescence staining was performed as described earlier (Mohapatra *et al.*, 2015). The expression of CDX2, dolichos biflorus agglutinin (DBA) and cytoskeletal proteins (keratin, vimentin and tubulin) was examined by immunofluorescence staining. TE cells were fixed with 100% ice-cold methanol, and the cells were kept at −20°C for 15 min that had been cultured in 4-well plates were washed three times with Dulbecco's Phosphate Buffered Saline (DPBS) and then permeabilized with 1% Triton X-100 in DPBS (DPBST) for 1 h. After thorough washing with DPBS, the cells were incubated with the blocking solution (5% BSA) for 1 h, followed by an overnight incubation at 4°C with the primary antibody which included anti-CDX2 (ready-to-use, AM392-10M, Bio-Genex Inc., San Ramon, CA, USA), anti-keratin (1:500, MAB1611, Millipore, Temecula, CA, USA), anti-vimentin (1:200, V6630, Sigma, USA) and anti-tubulin (1:400, T8328, Sigma, USA). For negative controls, the entire procedure was followed except that the primary antibody was replaced with mouse IgG. After three washings with DPBS containing 0.1% Triton X-100 (0.1% DPBST), the TE cells were incubated for 1 h with the appropriate fluorescein isothiocyanate-labelled secondary anti-mouse antibody (1:1000, F0257-.5ML, Sigma, USA) and Alexa Fluor 594-conjugated donkey anti-mouse IgG (H+L) secondary antibody (1:1000, A21203, Invitrogen, USA) for CDX2, diluted in DPBS. The cells were washed three times with 0.1% DPBST followed by nuclear staining with either Hoechst 33342 or propidium iodide. For the examination of DBA, a fluorescein-labelled primary antibody (1:200, FL1031, Vector Lab, USA) was used. The cells were then examined under a fluorescence microscope (Diaphot, Nikon, Tokyo, Japan) after the addition of antifade solution. Each experiment was repeated at least three times.

Quantitative real-time PCR (qPCR)

RNA was isolated from pools of 10 blastocysts each using an RNAqueous Micro Kit (Ambion, Austin, TX, USA) as per the manufacturer's protocol. RNA was isolated from TE cells using the TRIzol (15596026, Invitrogen, USA) method as per the manufacturer's protocol. Following DNase treatment, an RT reaction was performed for cDNA preparation using superscript reverse transcriptase III (18080051, Invitrogen, USA). Quantification of mRNA was carried out by qPCR using CFX 96 I Cycler (Bio-Rad, USA) as described earlier by Sandhu *et al.* (2023). The reaction

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Table 1. Developmental competence of parthenogenetic embryos produced using the zona-free method

Trials	Number of oocytes (n)	Number of oocytes cleaved	Cleavage n (%)	Number of blastocysts produced	Blastocysts n (%)
Trial 1	88	75	85.22	25	28.4
Trial 2	117	106	90.59	38	32.47
Trial 3	105	90	85.71	24	22.85
Total	310	271	87.2 ± 1.71[a]	87	27.9 ± 2.78[b]

Data from three trials.
Values are mean ± standard error of the mean.
Values with different superscripts ([a,b]) within the same row differ significantly ($P < 0.05$).

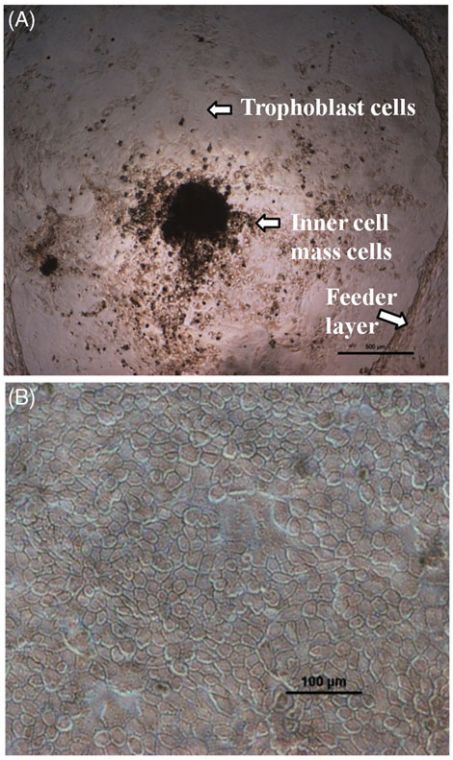

Figure 1. A parthenogenesis-derived blastocyst seeded on buffalo fetal fibroblast feeder layer, showing (A) a primary colony of trophoblast (TE) cells (40X) and (B) a monolayer of TE cells at passage 10.

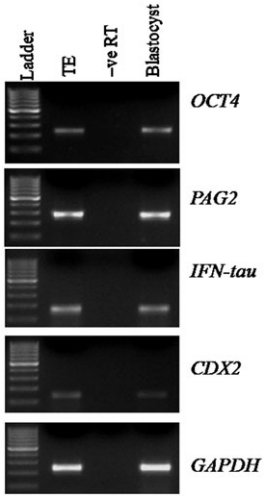

Figure 2. Expression of trophoblast (TE)-specific markers in TE cells and blastocysts examined by reverse transcriptase polymerase chain reaction.

mixture (10 µl) contained 5 µl SYBR Green master-mix (Maxima SYBR Green Mastermix, Thermo Scientific, USA), 0.2 µl of 10 µM of each primer and 2× diluted cDNA. Thermal cycling conditions consisted of initial denaturation at 95°C for 5 min, followed by 40 cycles of 15 s at 95°C, 15 s at the corresponding annealing temperature and 15 s at 72°C followed by 95°C for 10 s (Supplementary Table 1). All the primer pairs used were confirmed for their PCR efficiency, and specific products were checked by melt curve analysis and for the appropriateness of size by 2% agarose gel electrophoresis. Primer sequences are provided in the supplementary data (Supplementary Table 1). The expression data were normalized to the expression of Glyceraldehyde-3-Phosphate Dehydrogenase and were analyzed with CFX Manager software (Bio-Rad, USA). In all the experiments, three trials were carried out, each in duplicate.

Experimental design and statistical analysis

In Experiment 1, the parthenogenesis-derived blastocysts were cultured on a feeder layer in a TE culture medium to compare their attachment rate and the growth rate of primary colonies. For examining their morphology, the cells were regularly observed under a phase-contrast microscope.

In Experiment 2, TE cells were characterized by examination of the expression of TE-specific markers *OCT4*, *PAG2*, *IFN-tau* and *CDX2* by RT-PCR and of CDX2, DBA and cytoskeletal proteins (keratin, vimentin and tubulin) by immunofluorescence.

In Experiment 3, Day 8 parthenogenesis-derived blastocysts were seeded on MaxGel-coated dishes and were cultured in TE culture medium. The primary TE outgrowths were collected on days 0, 5, 9, 12 and 15 of culture. TE cell lines at passages 12–15, which had been derived from blastocysts produced by parthenogenesis were also included in the comparative study. The relative transcript level of *IFN-tau*, *FGFR2*, *DNMT1*, *DNMT3a*, *CDX2*, *PAG2*, *ELF5*, *ETS2*, *GATA3* and *GATA2* was compared among different groups.

Statistical analysis was carried out using Sigma Stat version 3.1 (Aspire Software International, VA, USA). The datasets were analyzed by one-way analysis of variance followed by the Holm–Sidak test. Percentage values were subjected to arcsine transformation prior to analysis. The differences were considered to be statistically significant at $P < 0.05$. Data were presented as mean ± standard error of the mean.

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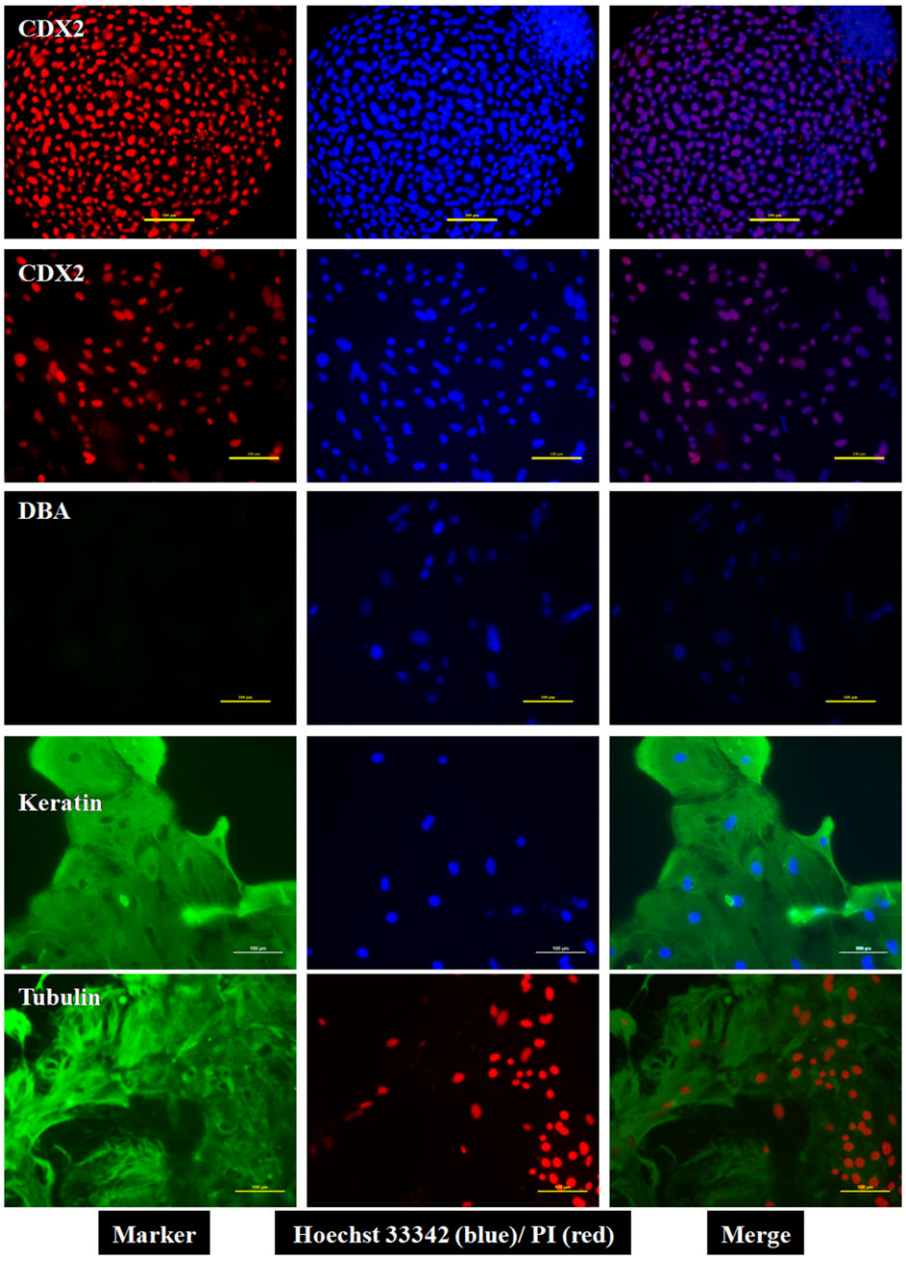

Figure 3. Immunofluorescence characterizations of the blastocyst with CDX2 (first row) and parthenogenesis-derived trophoblast cells with CDX2, DBA and cytoskeletal proteins.

Results

Out of a total of 310 oocytes used for zona-free parthenogenesis, 271 (87.2 ± 1.71%) cleaved, and 87 (27.9 ± 2.78%) reached the blastocyst stage (Table 1). Out of 44 blastocysts seeded on the BFF feeder layer, 21 (47.7%) got attached following which their diameter increased from 278 ± 25 µm to 2894 ± 225 µm from Day 0 to Day 7 of seeding (Figure 1A). Out of the blastocysts that got attached, 9 (42.8%), 4 (19.0%) and 2 (9.5%) cell lines could survive up to passages 1, 5 and 10, respectively (Supplementary Table 2), whereas one cell line (Figure 1B) could survive up to passage 21.

In Experiment 2, the cells were found to express TE-specific markers *IFN-tau*, *CDX2*, *PAG2* and *OCT4* by RT-PCR (Figure 2). The mRNA expression of all these markers was present in both TE cells as well as blastocysts. The expression of TE-specific marker CDX2 was present in both blastocysts as well as in TE cells (Figure 3). The TE cells also exhibited the expression of cytoskeletal markers keratin and tubulin but no expression of DBA (Figure 3). There is no expression for vimentin in TE cells (data not shown).

In Experiment 3, when Day 8 parthenogenesis-derived blastocysts were seeded on MaxGel-coated dishes and the primary TE outgrowths seen coming out of attached blastocysts were collected on days 0, 5, 9, 12 and 15 of culture, several patterns emerged in the expression level of genes examined. The relative transcript level of epigenetics-related genes *DNMT1* and *DNMT3a*, TE-specific markers *CDX2*, *ELF5*, *ETS2* and *GATA2* and development-related gene *FGFR2*, which was high on Day 0, decreased ($P < 0.05$) during the course of culture (Figure 4). The relative transcript level either decreased to the level observed

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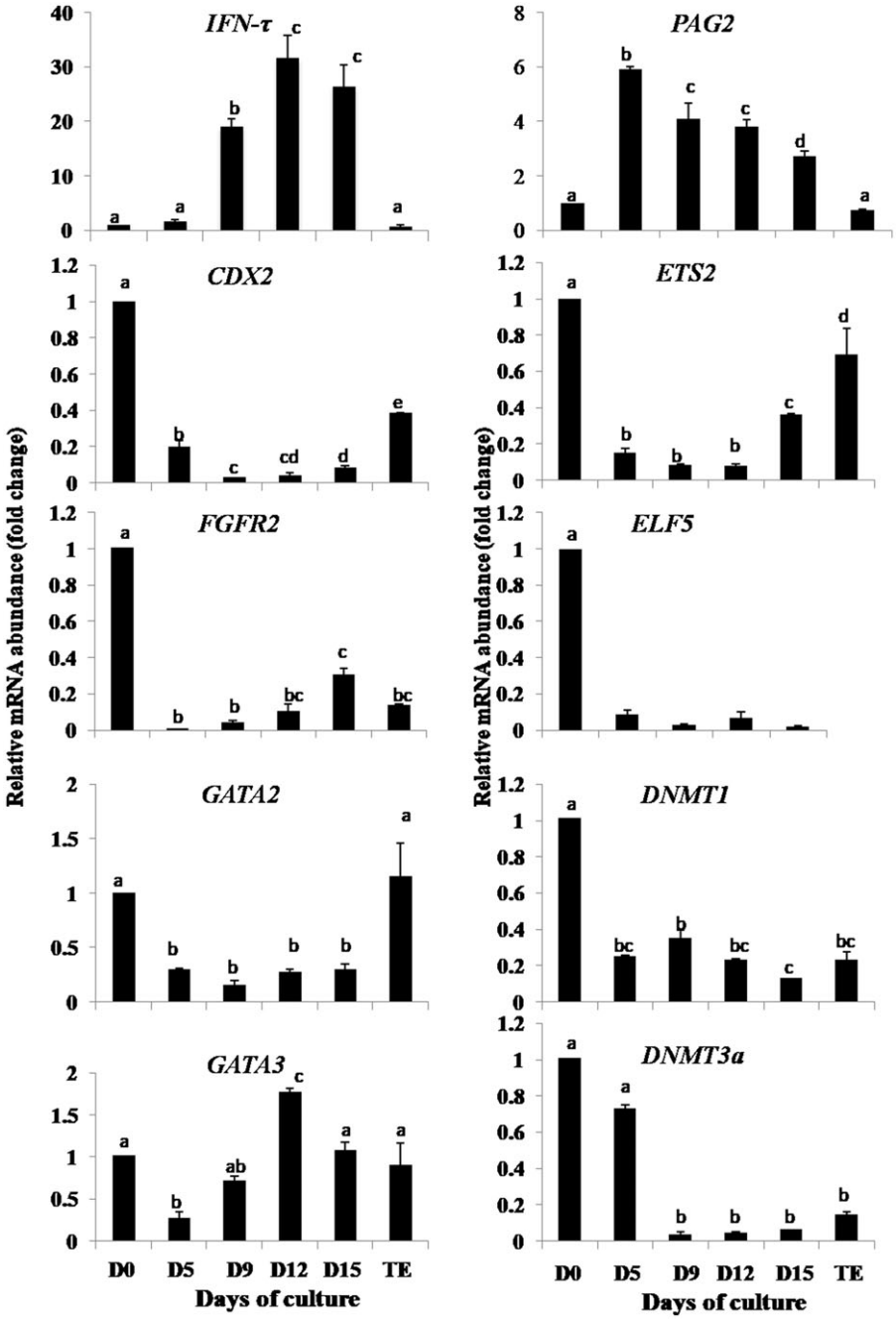

Figure 4. Expression levels of trophectoderm-related genes in blastocysts (D0), different days (5, 9, 12, 15) of the culture of trophoblast (TE) cells and TE cell line at passages 12–15 under feeder-free conditions derived from parthenogenesis. Bars with different superscripts differ significantly ($P < 0.05$) unless otherwise mentioned in the text.

in TE cells at passages 12–15 (*DNMT1*, *DNMT3a* and *FGFR2*) or was still significantly lower ($P < 0.05$) than that in TE cells at passages 12–15 (*ETS2*, *GATA2* and *CDX2*). In the case of *IFN-tau* and *PAG2*, the expression level increased significantly ($P < 0.05$) from Day 0 to Day 15 of culture and was significantly ($P < 0.05$) higher at Day 15 than that observed in TE cells at passages 12–15. In the case of *GATA3*, the expression level increased significantly ($P < 0.05$) from Day 0 to Day 12 of culture and then decreased ($P < 0.05$) to the level observed in TE cells at passages 12–15.

Discussion

Buffalo TE cells share many similar characteristics with cattle TE cells, including a flat, cuboidal appearance, granular cytoplasm and several lipid droplets (Mohapatra *et al.*, 2015; Shimada *et al.*, 2001). However, there are also some key differences between the two cell types. For example, buffalo TE cells cannot be completely dissociated by trypsinization, while cattle TE cells can. These differences may be due to species variation or differences in the origin of the cells. Another common feature was that following continuous culture, fluid accumulated under the monolayer resulting in the formation of a dome-shaped structure. In addition, the formation of distinct fluid-filled vesicles was also seen. The vesicles dissociated spontaneously from the colony and floated freely in the medium but became attached after being transferred to a new dish following which fresh outgrowths grew from the seeded vesicles. Porcine TE cells display a prominent nucleus and prominent lipid-containing vesicles but do not form fluid-filled

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vesicles (Flechon *et al.*, 1995). The morphology of buffalo TE cells derived from blastocysts derived from IVF and Handmade cloning (Mohapatra *et al.*, 2015) is similar to that of blastocysts derived from parthenogenesis. The cells were found to express TE-specific markers *IFN-tau*, *CDX2*, *PAG2* and *OCT4* by RT-PCR in blastocyst as well as TE cells. Following immunofluorescence staining, parthenogenetic embryo-derived buffalo TE cells expressed the proteins keratin and tubulin but lacked vimentin. This finding aligns with observations made by our previous study, where similar protein expression patterns in buffalo TE cells derived from IVF embryos were observed. Buffalo TE cells express cytokeratin but not vimentin, indicating that TE cells across several species are of epithelial and not fibroblast origin. The expression of CDX2 was present in both blastocysts and TE cells. Expression of Dolichos biflorus agglutinin (DBA) was not seen in immunofluorescence. DBA is exclusively bound to both the surface membrane and cytoplasm of binucleate cells. In contrast, mononucleate epithelial cells and fibroblasts did not show any DBA binding. Interestingly, these DBA-positive binucleate cells were fully mature, produced PL and lacked cytokeratin in their cytoplasm (Nakano *et al.*, 2002). It indicates our cells are not differentiated cells as they are cytokeratin positive and DBA negative.

Ortega *et al.* (2022) also observed the expression of genes like IFNT2, GATA2, GATA 3, PAG2 and HAND1 in a bovine TE cell model. Furthermore, buffalo TE cells express specific markers like CDX2, PAG2 and *IFN-tau*, confirming their trophectoderm identity. CDX2 expression, confirmed in blastocysts and TE cells by immunofluorescence, serves alongside GATA3 as a powerful duo for distinguishing TE from ICM cells across various species. This partnership, highlighted by Deb *et al.* (2006), Kuijk *et al.* (2008) and Home *et al.* (2009), remains relevant despite diverse regulatory pathways governing ICM/TE identity (Berg *et al.*, 2011). In the case of *IFN-tau* and *PAG2*, the expression level increased significantly ($P < 0.05$) from Day 0 to Day 15 of culture and was significantly ($P < 0.05$) higher at Day 15 than that observed in TE cells at passages 12–15. Interferon tau and PAG2 together play an important role in the establishment and maintenance of pregnancy in ruminants. *IFN-tau* suppresses the immune response and prevents PGF2α production, which allows the embryo to implant in the uterus. PAG2 promotes trophoblast cell proliferation and differentiation, which are necessary for placental development. The relative transcript level either decreased to the level observed in TE cells at passages 12–15 (DNMT1, DNMT3a and FGFR2) or was still significantly lower ($P < 0.05$) than that in TE cells at passages 12–15 (ETS2, GATA2 and CDX2). CDX2 and Ets-2 act together to directly activate the IFNτ promoter, while GATA2 and GATA3 likely contribute to the overall regulatory network controlling IFNτ expression (Saadeldin *et al.*, 2011). In the case of *GATA3*, the expression level increased significantly ($P < 0.05$) from Day 0 to Day 12 of culture and then decreased ($P < 0.05$) to the level observed in TE cells at passages 12–15. No previous study has examined gene expression during placenta development in this way. This approach yields crucial information about the dynamics of gene regulation during the period.

Parthenogenesis was employed for studying the developmental competence of oocytes obtained from repeat breeder Murrah buffaloes compared with normal cyclic, taking parthenogenesis as a tool to validate the oocyte competence (Kumar *et al.*, 2024). The study of buffalo trophoblast cells, derived from parthenogenetic embryos, holds immense promise for breakthroughs in animal biotechnology. However, further exploration is crucial to unlock its full potential. Delving deeper into the intricate signalling pathways that govern trophoblast cell differentiation and function is paramount. By unravelling these mechanisms, researchers can gain invaluable knowledge about placental biology in buffaloes without involving sire factor. This, in turn, could pave the way for enhanced reproductive efficiency in these animals. Additionally, insights gained from buffalo trophoblast research might even translate to advancements in human health and regenerative medicine, further widening the impact of this exciting field.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0967199424000339.

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Competing interests. The authors declare that no competing interests.

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