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Synergistic effect of basic fibroblast growth factor (bFGF) and epidermal growth factor on derivation of camel (*Camelus dromedarius*) trophoblast stem cells

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Short Communication

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Summary

This study aimed to optimize the derivation of trophectoderm from *in vitro*-produced camel embryos under feeder-free culture conditions using the basement membrane matrix Matrigel. Trophoblastic vesicles were obtained through mechanical microdissection of *in vitro*-produced camel (*Camelus dromedarius*) embryos. Supplementing the culture medium with 10 ng/ml of epidermal growth factor and 10 ng/ml fibroblast growth factor improved the attachment and subsequent outgrowths of cultured trophoblastic vesicles when compared with the control group and the groups supplemented individually with each growth factor. The expression levels of pluripotency genes octamer-binding transcription factor 4 (*Oct4*), sex determining region Y-box 2 (*Sox2*), myelocytomatosis proto-oncogene (c-*Myc*) and anti-apoptotic gene B-cell lymphoma 2 (*Bcl2*) were increased in trophoblastic vesicles supplemented with both growth factors when compared with the control group. Conversely, both growth factors decreased the expression of apoptotic genes tumour protein p53 (*p53*) and Bcl-2-associated X protein (*Bax*). To the best of our knowledge, this may be the first report describing the derivation of trophoblast stem cells from *in vitro*-produced camel embryos.

Introduction

Preimplantation embryo-derived stem cells are a good tool for studying early developmental biology and for biomedical research and genetic engineering in mammalian species (Blomberg & Telugu, 2012). Trophoblast stem cells are the first cells to differentiate from the embryo, have oligopotent stem cell characteristics, contribute to the formation of the fetal parts of the placenta and fetal membranes, and have the capacity to self-renew indefinitely (Latos & Hemberger, 2016; Peter *et al.*, 2017). Camel (*Camelus dromedarius*) is an important mammalian species in the Arabian Peninsula and some other African and Asian countries because it can tolerate harsh arid conditions with maintaining meat and milk production. Moreover, the differentiation process of trophoblasts remains poorly understood in the camel because only few studies have described the early embryonic development in this species. There have been few reports regarding the embryonic and adult stem cells from camel (*C. dromedarius*) (Mohammadi-Sangcheshmeh *et al.*, 2013; Saadeldin *et al.*, 2017a; Saadeldin *et al.*, 2018a, c). Trials have been performed to isolate *in vitro*-produced trophoblast cells from several mammalian species such as cow, buffalo, mouse, rabbit, and pig, either through the use of feeder cells (Talbot *et al.*, 2007) or feeder-free culture conditions (Shimada *et al.*, 2001; Wutz *et al.*, 2011; Kubaczka *et al.*, 2014; Dean *et al.*, 2015; Saadeldin *et al.*, 2015). While, derivation of camel trophoblast and embryonic stem cells has been reported using *in vivo*-derived camel embryos (Saadeldin *et al.*, 2017a); no reports have been declared regarding the isolation of trophoblast stem cells from *in vitro*-derived embryos.

Several trials have been reported on the beneficial effects of various growth factor supplementation on the development of preimplantation embryos (Lee & Fukui, 1995; Ahumada *et al.*, 2012; Pan *et al.*, 2015). A few studies have reported the effect of growth factors on embryo-derived outgrowths in mice (Haimovici & Anderson, 1993), while no reports have been declared regarding the effects of growth factors on trophoblast derivation from camels.

Therefore, the current study aimed to optimize the conditions for the successful culture of trophoblast cells from *in vitro*-derived camel embryos through supplementation of culture medium with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF).

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Material and methods

Unless otherwise stated, all the chemicals used were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

Camel ovaries were collected from a slaughterhouse in Riyadh and transported in 0.9% (v/v) saline solution (NaCl) at 30 to 33°C to the laboratory within 4 to 6 h. Antral follicles (2–8 mm in diameter) were aspirated and cumulus–oocyte complexes (COCs) with evenly granulated cytoplasm and enclosed in more than three layers of compact cumulus cells were selected. COCs were washed three times with HEPES-buffered tissue culture medium-199 (TCM-199) supplemented with 2 mM sodium bicarbonate, bovine serum albumin 0.1%, and 5 ng/ml gentamycin sulfate. COCs were *in vitro* matured in 100 µl drops of bicarbonate-buffered TCM-199 supplemented with 10% camel follicular fluid, 10 µg/ml follicle stimulating hormone (FSH), 10 µg/ml luteinizing hormone (LH), 10 ng/ml EGF, 0.3 µM cysteamine, 0.15 mg/ml L-glutamine, and 50 µg/ml gentamycin sulfate at 38.5°C in a humidified atmosphere of 5% CO_2 in air for 30 h (Yaqoob *et al.*, 2017). The culture medium was overlaid with mineral oil. After maturation, cumulus cells were stripped from oocytes by pipetting in 1 mg/ml hyaluronidase in HEPES-buffered TCM-199 and washed three times in TCM-199 supplemented with 10% fetal bovine serum (FBS). Cumulus-free oocytes extruding the first polar body (around 65% of total COCs) were activated in TCM-199 supplemented with 10% FBS and 5 µM ionomycin for 5 min in a dark chamber, as previously described (Saadeldin *et al.*, 2017b). The parthenogenetically activated oocytes were cultured in microdrops of KSOMaa medium (one oocyte/5 µl) overlaid with oil in a humid atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C for 8 days until blastocyst formation and hatching. Blastocyst formation was about 20% and hatching was about 50% of the total blastocysts.

Under a stereomicroscope and by using a microblade, mechanical isolation of trophoblast and inner cell mass from the hatched embryos was performed as previously described (Strom *et al.*, 2007) with modifications. Briefly, a microblade was used to dislodge the inner cell mass cell dark clumps from the trophectoderm and then the dislodged trophoblast portions were cultured in culture medium for 24 h to form trophoblastic vesicles (TVs). TVs ($n = 48$) were randomly divided in to four groups; 12 TVs from each group in four replicates (three TVs each). TVs were placed on 4-well dishes (Nunclon Surface, ThermoFisher Scientific, Waltham, MA, USA) freshly coated with basement membrane matrix (Matrigel, BD Biosciences) (Armant, 2006; Saadeldin *et al.*, 2015) to maintain feeder-free conditions. Culture medium of TVs was either supplemented with 10 ng/ml EGF (EGF group), 10 ng/ml FGF (bFGF, Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany) (FGF group), or 10 ng/ml EGF + 10 ng/ml FGF (EGF + FGF group). Plain culture medium was used for the control group.

TVs culture medium was comprised of Dulbecco's modified Eagle's medium supplemented with 1% non-essential amino acids, 2 mM L-glutamine, 1% insulin-transferrin-selenium, 0.1 mM β-mercaptoethanol, 10% FBS, and 1 mg/ml gentamycin. Culture was performed in a humid atmosphere of 5% CO_2 at 38.5°C.

Trophoblast vesicle attachment to Matrigel and cuboidal cells outgrowths of trophoblasts were recorded for each treatment. Trophoblast passaging was performed through mechanical chopping and culturing over freshly prepared Matrigel, as previously mentioned (Saadeldin *et al.*, 2017a).

Total RNA was isolated from the control and FGF + EGF-supplemented TVs (three triplicates, five each) using a total RNA isolation kit (Intron Biotechnology, Daegu, Korea). Total RNA concentration and quality were determined using a NanoDrop 2000 spectrophotometer (ThermoFisher). Reverse transcription (RT) was performed to generate complementary DNA and relative quantification of mRNA transcripts was determined by relative quantitative polymerase chain reaction (qRT-PCR) according to the method described by (Saadeldin *et al.*, 2017a). Normalization to the reference gene *GAPDH* was performed and the fold change and relative quantification of *Oct4*, *Sox2*, *Klf4*, c-*Myc*, *Cdx2*, *Krt8*, *p53*, *Bax*, and *Bcl2* transcripts were carried out using the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008). In all assays, non-template control (NTC) and reactions without RT resulted in negative amplification. Expression of each transcript in control trophoblast vesicles was set as an arbitrary unit to calculate the fold-change in the treated groups. Details on primers and approximate product size are listed in Table 1. Five biological replicates and three technical replicates were used.

Data of blastocyst attachment and trophoblast outgrowths were calculated as percentages and analyzed with the chi-squared test. Data of qRT-PCR were expressed as the mean ± SEM and compared by Student's *t*-test using the SAS program (Cary, NC, USA). Statistical significance was considered when $P < 0.05$.

Table 1. Primers used for relative quantitative PCR

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Product size (bp)[a]	Reference
Pou5f1 (Oct4)	CGAGAGGATTTTGAGGCTGC	GGGGAGTACAGTGTGGTGAA	125	Saadeldin *et al.* (2018c)
Sox2	AGCTCGCAGACCTACATGAA	TGGGAGGAAGAGGAAACCAC	146	Saadeldin *et al.* (2018c)
Myc	GGGCTAAGTTGGACAATGGC	TTCAGCTCGTTCCTCCTCTG	142	Saadeldin *et al.* (2018c)
Klf4	CATCAGCCTCATCCTCGTCT	ATTCCCGACCATAGAGCAGG	147	Saadeldin *et al.* (2018c)
Krt8	AAACTGAAGCTGGAGGTGGA	TCCAGCTCCACCTTGTTCAT	161	Saadeldin *et al.* (2017a)
Cdx2	AACCGCAGAGCAAAGGAAAG	CAGGGAAGACACAGGACTCA	144	Saadeldin *et al.* (2017a)
p53	AGCTCCTCTCCACCACAAAA	GTGAGCCTTGTTTTCCCCTG	159	Saadeldin *et al.* (2018b)
Bax	AGATCATGAAGACAGGGGCC	GCGATCATCCTCTGCAACTC	190	Saadeldin *et al.* (2018b)
Bcl2	CAGGCTCAACGTCGAATCAG	TAGGTGGGGCTTGGCAATTA	151	Saadeldin *et al.* (2018b)
GAPDH	TGCTGAGTACGTTGTGGAGT	TCACGCCCATCACAAACATG	134	Saadeldin *et al.* (2017a)

[a]The melting curve for each primer was evaluated by ViiA™ 7 apparatus-associated software and the product size was confirmed by gel electrophoresis of PCR products on agarose 1.5% referred by a 1 kb DNA ladder.

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Table 2. Effects of EGF and/or FGF on trophoblastic vesicle development

	Control	FGF	EGF	FGF + EGF
Number*	12	12	12	12
Attachment%	8.3%^{d} (1)	16.6^{c}%	50%^{b} (6)	75%^{a} (9)
TE growth%	0%^{c} (0/1)	50%^{b} (1/2)	50%^{b} (3/6)	77.7%^{a} (7/9)
Passage number	0	0	0	5

*The total number of four replicates, three TVs each.
TE. Trophoblast outgrowths.
^{a,b,c}Values carrying different superscripts are statistically different at $P < 0.05$.

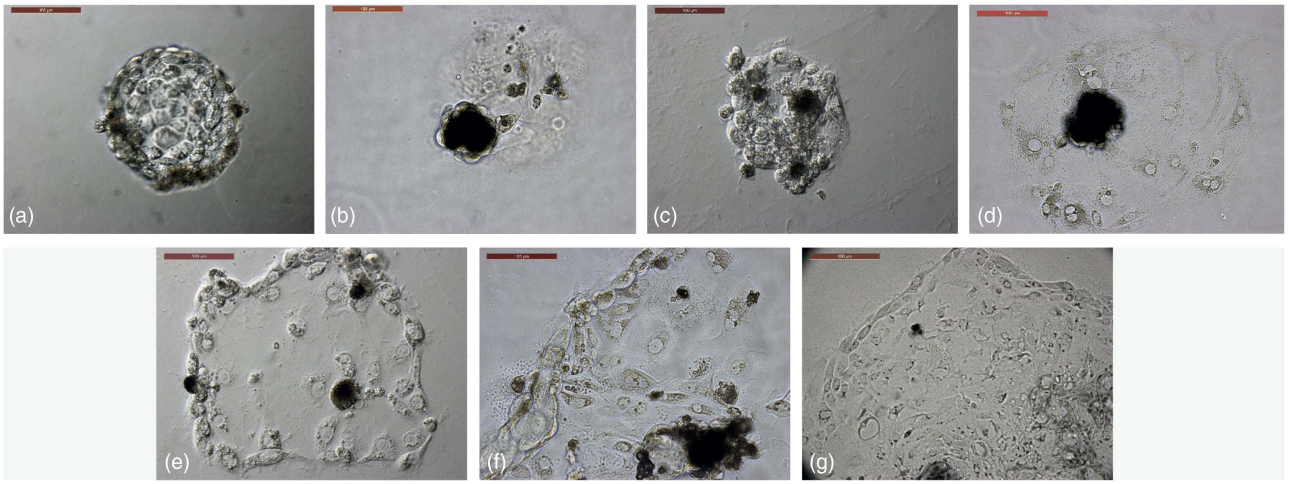

Figure 1. Trophoblast derivation from *in vitro*-produced camel embryos. (a) Trophoblastic vesicles (TVs) were cultured in 4-well dishes coated with freshly prepared Matrigel. (b) TVs growth in plain culture medium as a control group showing a lack of outgrowths. (c) TVs growth in EGF-supplemented culture medium, showing attachment with a lack of expanding outgrowths. (d) TVs growth in bFGF-supplemented culture medium, showing trophoblast outgrowths with a few cells that could not be passaged. (e–g) TVs growth in combined supplementation of culture medium with EGF and FGF, showing trophoblast outgrowths in primary culture – first passage and fifth passage, respectively. Scale bar represents 100 µm.

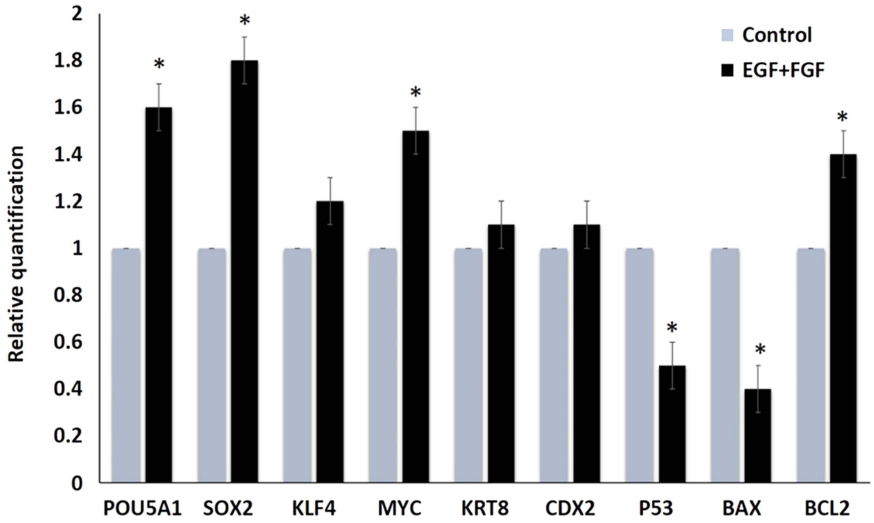

Figure 2. Relative quantification (fold change) of different transcripts after culturing TVs in plain culture medium (control) and medium supplemented with EGF and FGF. Asterisk (*) indicate significant difference at $P < 0.05$.

Results and discussion

TVs co-supplemented with EGF and FGF showed significant increases in attachment to Matrigel and trophoblast outgrowths, passaged to the fifth passage, and maintained the morphological criteria of the trophoblasts (Table 2 and Fig. 1). Therefore, culture medium co-supplemented with 10 ng/ml EGF and 10 ng/ml bFGF showed better developmental characteristics of cultured TVs over Matrigel basement membrane matrix when compared with the control group and the groups supplemented with each alone.

Additionally, combined supplementation of EGF and bFGF resulted in significant increases in pluripotency markers Pou5fa1 (Oct4), Sox2, and Myc when compared with the control group

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(1.6-, 1.8-, and 1.5-fold increases, respectively; Fig. 2). These increments might be required for the stemness criteria of the embryonic trophoblasts. Additionally, combined supplementation resulted in increased expression (1.45-fold) of the anti-apoptotic gene *Bcl2* and decreased expression of apoptotic genes *Bax* and *p53* (i.e. 2.5- and 2-fold increase in the control group, respectively). The *Bax*/*Bcl2* ratio was significantly decreased in combined growth factor-supplemented TVs when compared with the control group (0.28 vs. 1; $P < 0.05$). These changes might indicate the cellular death and replication defects that were observed in the control group (Fig. 2).

Studies have demonstrated the supporting role of bFGF in the derivation and growth of trophoblast stem cells in mice (Tanaka *et al.*, 1998) and humans (Kunath *et al.*, 2014). Recently, Okae *et al.* (2018) showed that EGF was essential for derivation and long-term maintenance of proliferative human trophoblast stem cells.

The current results reflect the first trial to optimize trophoblast culture from *in vitro*-derived camel embryos in feeder-free culture conditions by a synergistic effect between EGF and bFGF. This study provides a paradigm to further understand the stemness properties of trophoblasts derived from *in vitro*-produced camel embryos.

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Conflict of interest. The author declares that there is no conflict of interest that can be perceived as prejudicing the impartiality of the research reported.

Ethical standards. Not applicable.

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