Cell and Tissue Research (2021) 385:265–275
https://doi.org/10.1007/s00441-021-03418-7

REGULAR ARTICLE

Gene expression of bovine endometrial epithelial cells cultured in matrigel

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Received: 7 May 2020 / Accepted: 15 January 2021 / Published online: 10 April 2021

Abstract

Glandular epithelial cells (GE) in the endometrium are thought to support the elongation and survival of ruminant embryos by secreting histotrophs. In the present study, the gene expression of bovine endometrial epithelial cells cultured in matrigel was analyzed and examined whether it could be an in vitro model of GE. Bovine endometrial epithelial cells (BEE) and stromal cells (BES) were isolated from the slaughterhouse uteri and cultured in DMEM/F12 + 10% FBS. BEE showed the gland-like structure morphological changes when cultured in 15% matrigel but could not be identified in higher concentrations of the matrigel (30% or 60%). The expression of typical genes expressed in GE, *SERPINA14* and *GRP*, was substantially high in matrigel-cultured BEE than in monolayer ($P < 0.05$). P4 and INFα have no significant effect on the *SERPINA14* expression of BEE cultured in matrigel without co-culture with BES. On the other hand, when BEE were co-cultured with BES in matrigel culture, the expression of *FGF13* was increased by the P4 treatment ($P < 0.05$). Furthermore, *SERPINA14* and *TXN* expressions were increased by P4 + IFNα treatment ($P < 0.05$). These results demonstrate the appropriate conditions for BEE to form glandular structures in matrigel and the effect of co-culture with BES. The present study highlighted the possible use of matrigel for the culture of BEE to investigate the expression of cell-specific glandular epithelial genes as well as P4 and type-I IFN as factors controlling endometrial function during the implantation period.

Keywords Bovine · Endometrium · Epithelial cells · Gene expression · Matrigel

Summary sentence: Gene expressions of bovine endometrial epithelial cells cultured in matrigel were analyzed. The cells showed the gland-like structure when cultured in 15% matrigel. The expression of *FGF13*, *SEPARINA14*, and *TXN* was increased when the cells were co-cultured in matrigel with endometrial stromal.

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Introduction

There are two types of epithelial cells in the endometrium: luminal epithelium (LE) lined as a single layer facing the uterine lumen and glandular epithelium (GE) forming the uterine gland. GE originates from the LE postpartum, which eventually forms a network of coiled and slightly branched tubules extending throughout the stroma (Spencer et al. 2019). Each type of epithelial cells has a different role in uterine function. Studies with sheep and mice devoid of uterine glands provided direct evidence that GE-derived histotroph is required for embryo implantation and establishment of pregnancy (Filant and Spencer 2013; Cooke et al. 2012; Gray et al. 2001). It has been reported in studies using the uterine gland knockout sheep that embryo development and the hatching of blastocysts can normally occur without the glandular epithelium, but subsequent survival and elongation of the embryos are impaired (Gray et al. 2002). However, the mechanism in the histotrophic support of GE for the embryo elongation and

survival remained to be clarified. In cattle, the embryonic loss frequently occurs prior to maternal recognition of pregnancy at approximately day 16 following conception (Diskin et al. 2006; Diskin and Morris 2008). Since this period coincides with the timing of embryo elongation, functional analysis of GE has an important meaning for improving the conception rate.

Investigations of the function of endometrial cells in modulating the local physiology of pregnancy have benefitted from cell culture (Davis and Blair 1993). However, epithelial cells in monolayer culture exhibit changes in morphology and may have altered expression of certain genes (Gospodarowicz et al. 1980; Lee et al. 1984; Cooke et al. 1986; Thomas et al. 1992). Previous studies indicated that matrigel, extracellular matrix (ECM) component which derived from Engelbreth-Holm-Swarm mouse sarcoma cells, can restore the polarity and secretion of various epithelial cells in vitro, including the uterine epithelium from rodents (Eritja et al. 2010) and human (Rinehart et al. 1988). Haeger et al. (2018) reported that bovine endometrial gland cell line retained an in vivo-like phenotype and responded to IFNτ stimulation after steroid pre-incubation using matrigel. These results further suggest that matrigel culture of the endometrial epithelial cells may be suitable as a model for GE. However, these reports were mainly analyzed on cell polarity and morphological changes, and it is not clarified whether their functions including gene expression are similar to those of GE in the uterus. In order to use matrigel culture of endometrial epithelial cells as an in vitro model, it is necessary to clarify those function and gene expression in detail.

The purpose of this study is to analyze the gene expression of bovine endometrial epithelial cells cultured in the matrigel. The effect of matrigel culture was verified by comparing the expression of glandular epithelial cell-specific genes with monolayer culture cells. The effects of P4 and type-I IFN as factors regulating endometrial function during the implantation period were analyzed. Furthermore, the effect of co-culture with endometrial stromal cells in matrigel culture was also verified.

Materials and methods

Animals and tissue collection

Cows were superovulated by using FSH (Antorin R-10; Kyoritsu Seiyaku Co., Tokyo, Japan) on day 10 of the estrus. Injections of FSH were administered twice a day, i.e., in the morning and in the evening for three days with gradually decreasing dosage (5, 3, and 2 IU, at the days 1, 2, and 3, respectively). Besides, 2 ml of prostaglandin F2α (PGF2α) (Resipron-C; ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) was also injected in the morning of the third day. Then, 48 h after PGF2α injection, animals were observed for estrus behavior, and only those noted in standing estrus (designated as day 0 of pregnancy) were inseminated with cryopreserved sperm from a bull used for breeding. Cows were then slaughtered on day 18 of pregnancy, and the presence of the elongated conceptus in the uterus was confirmed. After slaughtered, the reproductive tract of each cow was collected and trimmed of extraneous tissue. The uterus was immersed in 70% ethanol and then washed two times with PBS (Dulbecco's PBS(-); Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 38.5 °C. The uterine horns which appropriately developed elongated conceptus on day 18 were further processed as a pre-implantation group ($n = 5$).

Bovine uteri from estrus cycle groups, follicle stage ($n = 5$) and luteal stage ($n = 5$), were also collected from slaughterhouse. The stage of the estrus cycle was determined by the ovarian morphology (Ireland et al. 1980). During the follicle stage, the ovary contains at least one large follicle, and a regressed corpus luteum with no vasculature was visualized on its surface. In contrast, during the luteal stage, a corpus luteum is fully formed with vasculature visible around its periphery.

The uterus was opened longitudinally, and samples were carefully cut from the lamina propria of endometrium with scissors and transferred into serum tubes (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). These samples were then stored at − 80 °C until further processing.

Culture of bovine endometrium cells

Bovine endometrial epithelial cells (BEE) and endometrial stromal cells (BES) were separated and purified from the endometrium of bovine uteri collected from the slaughterhouse, according to the protocol of Yamauchi et al. (2003). The endometrial tissues were surgically hashed with scissors, and the resultant tissues were incubated in culture medium (DMEM/Ham's F-12; NacalaiTesque, Inc., Kyoto, Japan) containing 0.1% (w/v) type-I collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 1% (w/w) antibiotic-antimycotic mixed stock solution (NacalaiTesque, Inc.) for 1 h at 37 °C. After incubation, tissue fragments were centrifuged at 300×*g* for 3 min with a Tabletop Centrifuge (Kubota 2410; Kubota Co., Tokyo, Japan) and washed with the culture medium for more two times. Then, the tissue fragments were plated in 75-cm^2 Cell Culture Flasks (TR6002; Nippon Genetics Co., Ltd., Tokyo, Japan) and cultured in the CO_2 incubator (Astec Co., Ltd., Fukuoka, Japan) at 37 °C in a humidified atmosphere of 5% CO_2. The primary culture cells were designated as population-doubling level (PDL) one, and cells were used at PDL three to five for further studies. To separate different types of cells, different

reactivities for trypsin were utilized; BES was detached from flasks with a lower concentration of trypsin, whereas BEE was detached with a higher level of trypsin.

Three-dimensional culture of bovine BEE in matrigel

To avoid the detachment of the gel from the plate due to shrinkage of cultured BEE, 40 µl of matrigel (Becton, Dickinson and Company, 354230) was coated at the bottom of each well of 96-well plate and incubated for 30 min at 37 °C. BEE were removed from the flask by using trypsin-EDTA solution (Nakarai), and the cells were washed by centrifugation (300×*g*, 3 min) with the culture medium for 3 times. The number of the cells was adjusted to 2.0×10^6 cells/ml of the medium. Then, the cell suspension and matrigel were mixed to adjust for the concentration of matrigel at 15, 30, and 60%. Sixty microliters of this mixture was put on each well coated with the matrigel and incubated for 1 h at 37 °C. After the incubation, 60 µl of the fresh medium was placed on each well, and the top of the medium, which did not include cells and matrigel, was changed every two days.

Co-culture of BEE and BES in matrigel

BES at concentration of 1.0×10^4 cells/well were cultured in each well of 96-well plate. After 1 day, 40 µl of matrigel was plated on the monolayer cultured BES; then, BEE cultured in matrigel at a concentration of 15% were plated on the gel as described above.

Treatment of cells with progesterone and interferon α

For the treatment of cells, 1 µM progesterone (P4, Sigma-Aldrich Co., Ltd., St. Louis, MO, USA) and/or 250 IU/ml human interferon α2 (IFNα; Pestka Biomedical Laboratories, Inc., Piscataway, NJ, USA) were supplemented

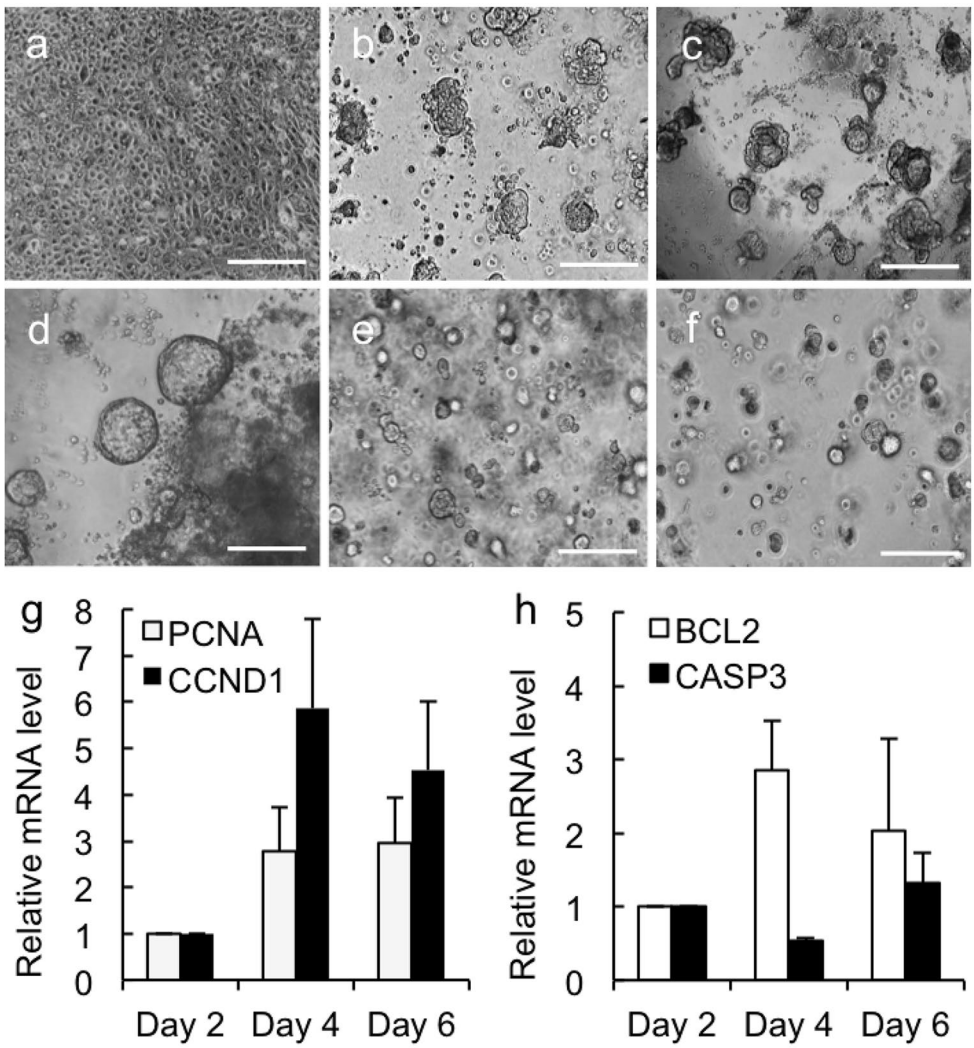

Fig. 1 Phase-contrast images **a**–**f** and gene expression **g** and **h** of BEE cultured in matrigel. Monolayer cultured BEE at 6 days **a**, cultured in 15% matrigel for 2 days **b**, 4 days **c**, 6 days **d**, 30% matrigel for 6 days **e**, and 60% matrigel for 6 days **f**, respectively. Scale bars represent 200 µm. Gene expression analysis of proliferation **g** and apoptosis **h**-related genes. BEE were cultured in matrigel for 2, 4, and 6 days. The mRNA expression was normalized to GAPDH and shown as means ± SEM relative to day 2 (= 1.0). Different superscripts in each panel indicate the significant differences ($P < 0.05$)

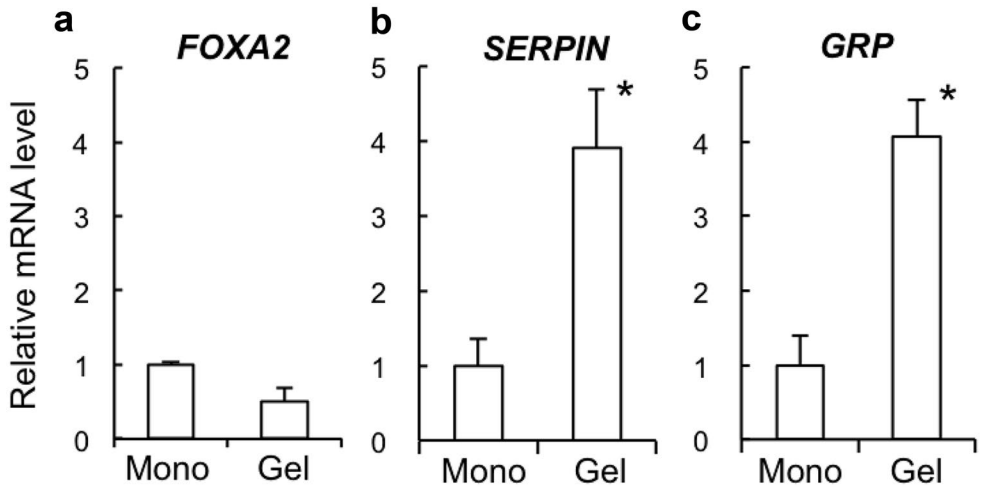

Fig. 2 Expression of glandular epithelial specific factors in bovine endometrial epithelial cells cultured in matrigel. The expression of *FOXA2* **a**, *SERPINA14* **b**, and *GRP* **c** in BEE cultured in matrigel (Gel) were compared with that of monolayer cultured BEE (Mono). Expressions of gene were analyzed by real-time qPCR. Each mRNA expression was normalized to *GAPDH* and shown as means ± SEM relative to the value of each monolayer (= 1.0). Asterisks indicate significant differences compared with each expression in monolayer ($P < 0.05$)

in the culture medium. Generally, to analyze the effect of P4 on the gene expression, the cells were treated for 24 h in culture (Rahman et al. 2020). Accordingly, P4 treatment was carried out for 24 h when culturing BEE alone (Fig. 5b). On the other hand, considering the interaction between BES and BEE, the treatment of the cells was further extended for 24 h, and the treatment was performed for a total of 48 h (Figs. 3a–f; 5c; and 6). Since P4 and INFα differ in the time required to induce gene expression, INFα treatment was performed only for the last 4 h of the P4 treatment (Fig. 5b, c). Therefore, in the co-culture experiment using P4 and INFα, the treatment duration was 48 h and 28 h, respectively.

Total RNA extraction, reverse transcription, and RT-qPCR

Cultured bovine endometrial cells in matrigel were recovered with BD cell recovery solution (BD Bioscience, San Jose, CA) according to the manufacturer's instructions. Briefly, cultured cells were washed with ice-cold PBS and scraped with the BD cell recovery solution on ice. Then, the cell suspension was transferred to the tube and centrifuged 3 times. The cell pellet was suspended in PBS and subjected for total RNA isolation, purification, quantification, and reverse transcription. During this process, mRNA of the BES and BEE was recovered together in a co-culture experiment.

Total RNA was extracted from endometrium tissues or cells by using the RNeasy Mini Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. All the reagents of reverse transcription were purchased from ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO Co., Osaka, Japan). To quantify the amount of total RNA extract, the optical density (260 nm) was determined with a NanoDropLite (Thermo Fisher Scientific Co., Waltham, MA, USA), and the RNA quality was assessed by spectrophotometric UV absorbance at 260/280 nm. Reverse transcription was performed using 6 ng of total RNA in a 10-µl reaction volume. RNA was incubated at 65 °C for 5 min and 4×DN Master Mix (added gDNA Remover) were mixed on ice prior. After 5 min incubation at 37 °C, the reaction liquid mixed with 5×RT Master Mix II on ice prior, and cDNA was synthesized according to the customized reaction condition (i.e., reverse transcription for 15 min at 37 °C and 5 min at 50 °C and inhibition reaction of the enzyme of reverse transcription for 5 min at 98 °C).

Reverse transcription PCR and real-time PCR were performed with Go Taq-Green Master Mix, 2× (Promega Corporation., Madison, WI), and THUNDERBIRD® SYBR qPCR Mix (TOYOBO) using an Mx3000P qPCR system (Agilent Technologies, Santa Clara, CA, USA), respectively. All primers were chosen based on primer design software from the national center for biotechnology information (NCBI). Specific primer sequences and size of resulting fragments for reference and target genes are shown in Table 1. All primers were validated before use (95–97%), and PCR amplification was conducted with an initial 2 min step at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The fluorescent SYBR Green signal was detected immediately after the extension step of each cycle, and the cycle at which the product was first detectable was recorded as the cycle threshold. The fluorescent SYBR Green signal was detected immediately after the extension step of each cycle, and the cycle at which

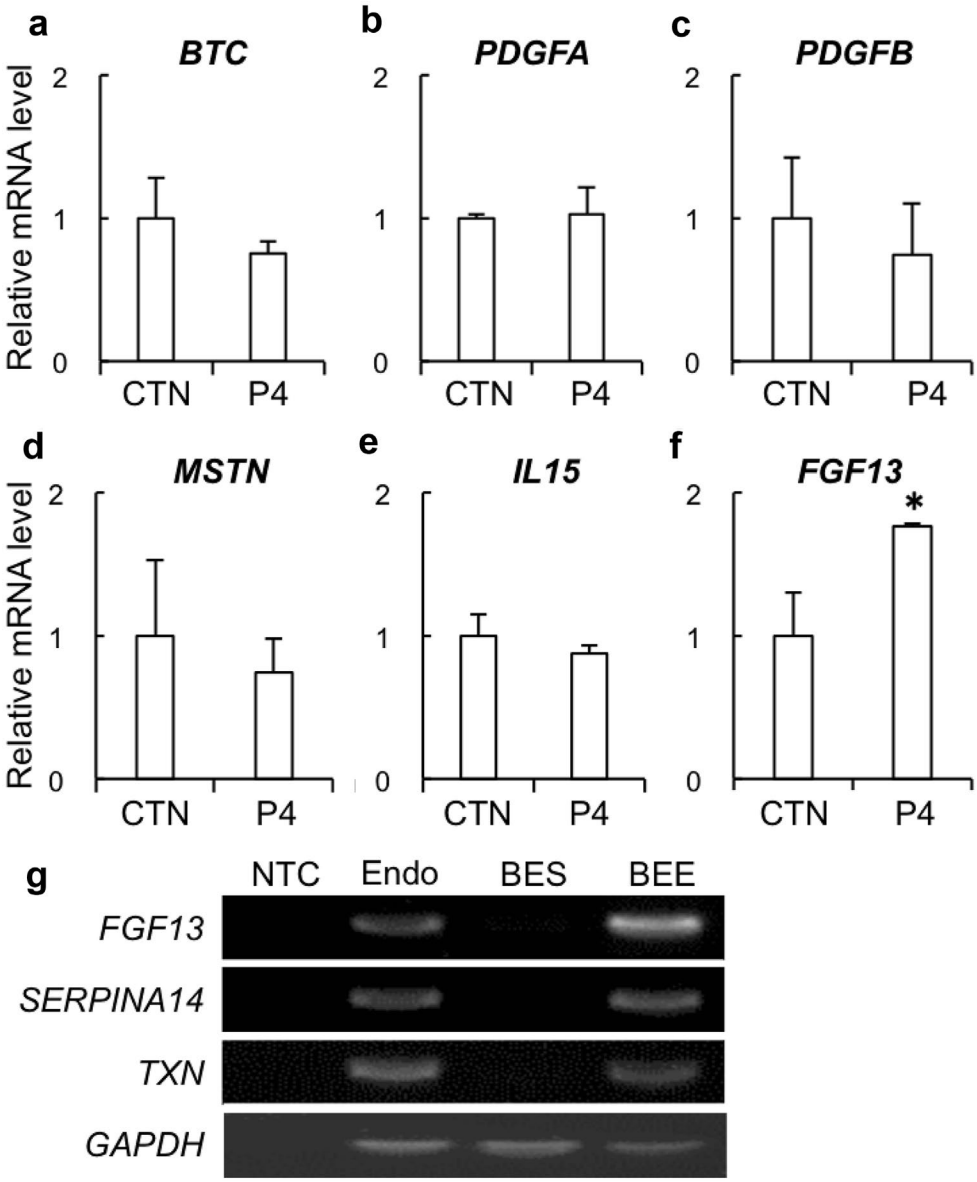

Fig. 3 Gene expression of BEE and BES cultured in matrigel. Effect of P4 on the gene expression in the co-culture of BEE and BES in matrigel **a**–**f**. Cells were treated with P4 for 48 h. The expressions of *BTC* **a**, *PDGFA* **b**, *PDGFB* **c**, *MSTN* **d**, *IL15* **e**, and *FGF13* **f** were analyzed by real-time qPCR. The mRNA expression was normalized to GAPDH and shown as means ± SEM relative to the control (CNT) (= 1.0). Asterisks indicate significant differences compared with each CNT ($P < 0.05$). P4, progesterone. Expression of *FGF13*, *SEPARINA4* and *TXN* in BES and BEE cultured separately in matrigel **g**. Gene expression was analyzed by RT-PCR. Bovine endometrial tissue (Endom.) at implantation stage was used as positive control. Negative control (NTC) included RNase-free water instead of cDNA

the product was first detectable was recorded as the cycle threshold. *GAPDH* served as an internal control and was used to normalize for differences in each sample.

Statistical analysis

Each experiment was repeated at least three times, and the results were expressed as a ratio against each control as mean ± SEM. Single-factor analysis of variance (ANOVA) was used to analyze the statistical differences where significances were considered at $P < 0.05$. Furthermore, the Student-Newman-Keuls test was used to compare two groups. Differences were considered to be significant at the level of $P < 0.05$.

Results

Morphological features and gene expressions of bovine endometrium epithelial cells cultured in matrigel

The bovine endometrial epithelial cells (BEE) were cultured in the matrigel with different concentration (15, 30 and 60%) and in an ordinary monolayer (Fig. 1a). On 2 days of culture, BEE was initiated to aggregate at 15% in-gel culture (Fig. 1b). Further, on the day 4 of culture, the aggregated BEE formed a circular or elliptical gland-like structure under the microscope in 15% in-gel culture (Fig. 1c). After 6 days of culture, elliptical gland–like structure found in increased

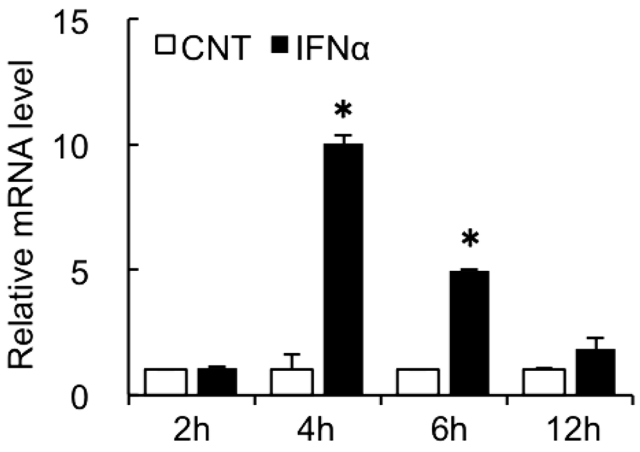

Fig. 4 Effect of IFNα on temporal change of *MX1* expression in BEE cultured in matrigel. The mRNA expression levels of *MX1* were determined by real-time qPCR and compared with the control (CNT) of the same culture period. BEE were treated with IFNα (100 unit/well) for 2, 4, 6, or 12 h. Data are expressed relative to the levels of the *GAPDH* and shown as means ± SEM which normalized to each CNT (= 1). Asterisks indicate significant differences compared with each CNT of the group ($P < 0.05$). IFNα, human interferon α2

form and vesicular morphology was also recorded (Fig. 1d). On the other hand, the morphological change of gland-like structure as observed in 15% in-gel culture could not observed in the cells cultured in matrigel at concentration of 30% (Fig. 1e) and 60% (Fig. 1f). Therefore, in the following experiments, BEE were cultured in matrigel at a concentration of 15% that formed a gland-like structure.

Biological activity of BEE associated with morphological changes, gene expression related to cell proliferation (*PCNA* and *CCND1*), and apoptosis (*BCL2* and *CASP3*) were examined over time. The expression of *PCNA* and *CCND1* increased more than 3-folds compared with 2 days of culture. However, no significant difference was observed due to large deviation (Fig. 1g). Similarly, *BCL2* and *CASP3* were also tended to increase after culture, but no significant difference was observed (Fig. 1h).

Expression of glandular epithelial specific factors in bovine endometrial epithelial cells cultured in matrigel

Gene expression of glandular epithelial specific factors, *FOXA2*, *SERPINA14*, and *GRP*, in bovine endometrial epithelial cells (BEE) cultured in matrigel was analyzed by real-time qPCR. The expression of *FOXA2* was not different between BEE cultured in matrigel and in monolayer (Fig. 2a). On the other hand, the expressions of *SERPINA14* and *GRP* were significantly high in BEE cultured in matrigel than that in monolayer ($P < 0.05$) (Fig. 2b, c).

Effect of P4 on the gene expression of endometrial cells co-cultured in matrigel

Effect of P4 on the expressions of *BTC*, *PDGFA*, *PDGFB*, *MSTN*, *IL15*, and *FGF13* in the co-cultured of BEE and BES in matrigel was analyzed by real-time qPCR (Fig. 3a–f). These genes were selected from genes that encode secretory proteins expressed in the endometrium on day 13 of pregnancy (Musavi et al. 2018), which is the beginning of elongation of the bovine embryo. The result showed that there was no difference in the gene expression of examined factors among the control and P4-treated groups, except *FGF13*. As a result of RT-PCR, *FGF13* was not expressed in BES in matrigel culture but was expressed only in BEE (Fig. 3g). The expression of *FGF13* in the co-cultured cells in matrigel was significantly high in P4-treated groups, approximately 1.8-fold, than that in the control (Fig. 3f).

Temporal change of MX1 expression after type-I IFN treatment in BEE cultured in matrigel

In order to clarify the timing of gene expression after type-I INF treatment, the expression of *MX1* in BEE cultured in matrigel was examined (Fig. 4). The results showed that the expression of *MX1* in BEE treated with IFNα for 4 h significantly increased approximately tenfold higher than that in the control group ($P < 0.05$). The expression at 6 h of the treatment also increased significantly approximately fivefold higher than that in the control group ($P < 0.05$). However, there was no difference after 12 h of the treatment. From this result, the subsequent IFN treatment experiment was performed for 4 h.

Expression of SERPINA14 in bovine endometrium and BEE

In bovine endometrial tissue, the expression of *SERPINA14* was most significant during the implantation stage compared with others (Fig. 5a). Expression of *SERPINA14* was 13-fold higher in luteal stage compared with that in follicle stage, but there was no difference between the follicle stage and luteal stage statistically. On the other hand, the expression of *SERPINA14* was significantly high approximately 85-fold higher in implantation stage compared with that in follicle stage.

Comparing the expression of BEE in monolayer and matrigel culture, *SERPINA14* expression of BEE was high when cultured in matrigel regardless of the addition of P4 and INF (Fig. 5b). It was significantly high in the control and INF-treated groups compared with the monolayer cultured cells ($P < 0.05$). In comparison with each culture condition

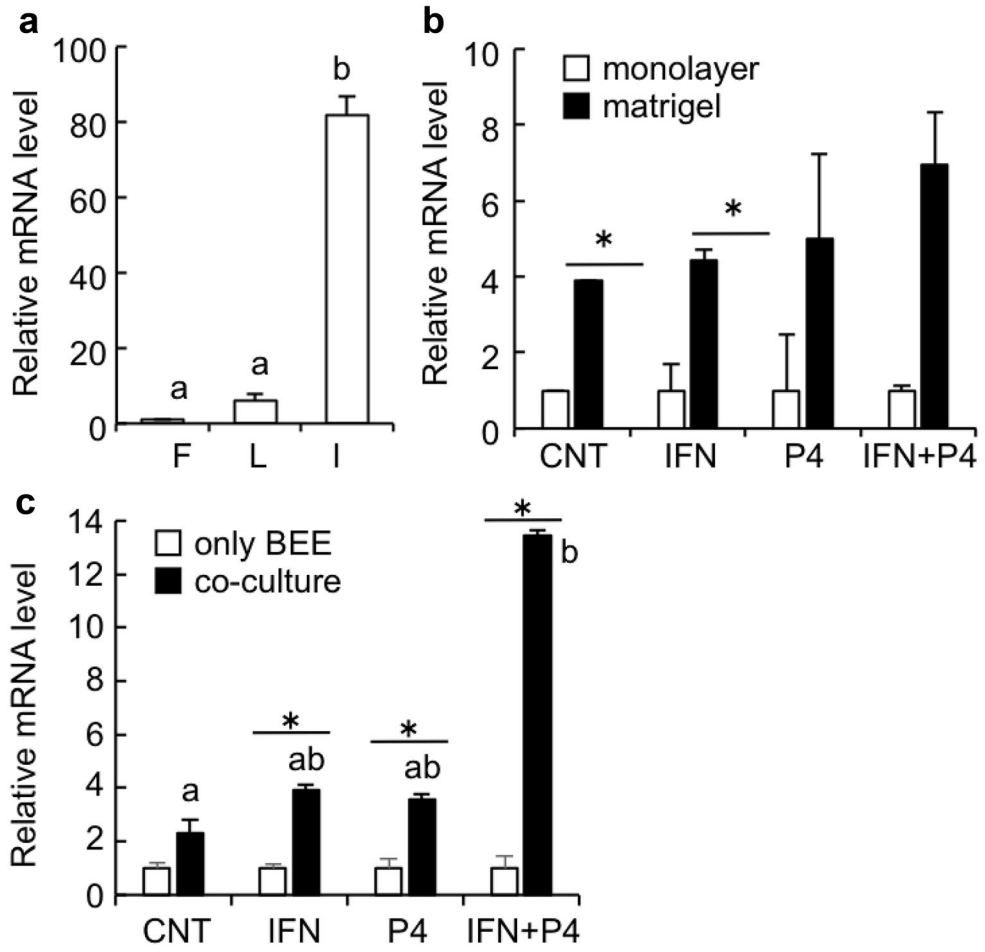

Fig. 5 Expression of *SERPINA14* in bovine endometrium tissue and epithelial cells in vitro. The expression of *SERPINA14* in endometrial intercaruncular tissues during estrus cycle and early pregnancy **a**. The mRNA expression was normalized to *GAPDH* and shown as means ± SEM relative to the level of the follicular phase (= 1). Different superscripts indicate the significant differences ($P < 0.05$). The expression of *SERPINA14* in BEE on monolayer (white bar) and in matrigel (black bar) **b**. The cells were treated for 24 h with P4 and for 4 h with INF. The mRNA expression was normalized to *GAPDH* and shown as means ± SEM relative to monolayer (= 1). Asterisks indicate significant differences compared with each monolayer of the group ($P < 0.05$). The expression of *SERPINA14* in BEE co-cultured without (white bar) or with BES (black bar) in matrigel **c**. The cells were treated for 48 h with P4 and for 28 h with INF. The mRNA expression was normalized to GAPDH and shown as means ± SEM relative to each expression in only BEE (= 1.0). Different superscripts in the co-culture groups indicate the significant differences ($P < 0.05$). Asterisks indicate significant differences compared with or without co-culture in each treatment group ($P < 0.05$). CNT, control; IFN, human Interferon α2; P4, progesterone

of monolayer and matrigel culture, the effect of P4 or INF on the expression of *SERPINA14* was not observed.

Similar experiment was conducted in a co-culture system with BES and compared with culturing only with BEE in matrigel (Fig. 5c). RT-PCR result reveals that *SERPINA14* not expressed in BES in matrigel culture, but only expressed in BEE (Fig. 3g). There was no difference in the expression of *SERPINA14* in the co-culture treated with P4 or IFN alone, compared with the control groups. In contrast, the expression significantly increased in groups treated with both P4 and IFN ($P < 0.05$). The expression of *SERPINA14* in co-culture treated with P4 or IFN alone was significantly higher, approximately fourfold, than that in BEE cultured in matrigel without co-culture with BES. Moreover, the expression of *SERPINA14* in co-culture treated with P4 and IFN found approximately 13-fold higher than BEE without the co-culture, while there were no differences in the expression between with or without co-culture with BES in the CNT groups.

Effect of type-I IFN and P4 on expression of the secreted factors in BEE co-culture with BES in matrigel

ALDOA and TXN are the factors that encode secretory proteins that are expressed in the endometrium on the

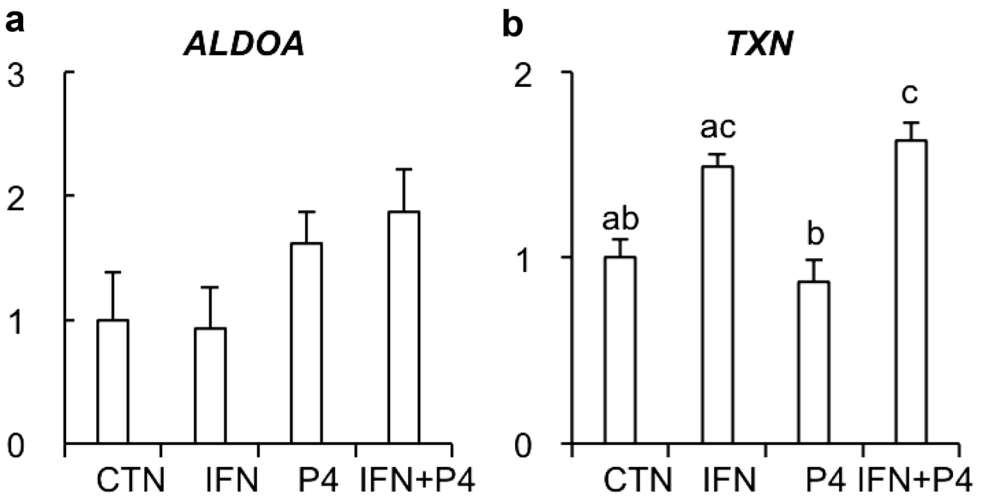

Fig. 6 Effect of IFN and P4 on the expression of *ALDOA* and *TXN* in the co-culture of BEE and BES in matrigel. The expressions of *ALDOA* **a** and *TXN* **b** were analyzed by real-time qPCR. The cells were treated for 48 h with P4 and for 28 h with INF. The mRNA expression was normalized to GAPDH and shown as means ± SEM relative to the control (CNT) (= 1.0). Different superscripts in each panel indicate the significant differences ($P < 0.05$). CNT, control; IFN, human Interferon α2; P4, progesterone

13th day of pregnancy (Musavi et al. 2018). There was no difference in the expression of *ALDOA* among the groups with different treatments in co-culture (Fig. 6a), while there was no difference in the expression of *TXN* in the co-culture treated with P4 or INFα alone compared with the control. However, the expression of TXN significantly increased in IFNα + P4–treated groups compared with the control ($P < 0.05$). Moreover, the expression in IFNα alone

Table 1 Sequences of primer pairs used for quantitative real-time PCR

Gene	Sequence	AT (°C)	PCR product (bp)
PCNA	F: AGGGCTTCGACACTTACCG R: ACGTGTCCGCGTTATCTTCAG	60	132
CCND1	F: CTCTCCTATCACCGCCTGAC R: CTTTGGGGTCCAGTTCTGC	60	142
BCLS	F: TGGATGACCGAGTACCTGAAC R: CAGGAGAAATCAAACAGGGGC	60	116
CASP3	F: CTGAGGGTCAGCTCCTAGCG R: CCAGAGTCCATTGATTTGCTTCC	60	187
FOXA2	F: CGAGGCCCACCTGAAGC R: TCGTAGGCCTTGAGGTCCAT	60	138
SERPINA14	F: AGTGACTTCAGACTGGTGCG R: GGGGTCAATCTTGGGAAGCA	60	90
GRP	F: ATTCCGAGGACGTCAGCAAC R: TTGCTTCTTTGAGGGAGGCG	60	104
BTC	F: ACCACACAACCAAAGCGAAG R: CCCAGCATAGCCTTCATCACA	60	135
PDGFB	F: CTGCTACCTGCGTCTGGTC R: CAGCCCCGTCTTCATCTACG	60	140
MSTN	F: AAGACGATGACTACCACGCC R: GGGTTTTCCTTCCACTTGCG	60	86
FGF13	F: TTTCCCGGGTCAAACTCTTCG R: GCCATCAATAGTTCCATCTGCC	60	137
TXN	F: TCCAACGTGGTGTTCCTTGA R: CACCCACCTTCTGTCCCTTT	60	115
ALDOA	F: GAGTCCTGGTGACGATTGCC R: CACCCACCTTCTGTCCCTTT	60	125
IL15	F: CGAGTCCTGGTGACGATTGCC R: TCCTCACATTGTTTGCATCCCA	60	114
PDGFA	F: CAACACGAGCAGCGTGAAG R: CCGCACCTGCACCTGTTTTA	60	136
GAPDH	F: CTGTGCTGTGCCAGCCGCAT R: GGCGACGATGTCCACTTTGCC	60	122

AT annealing temperatures, *F* forward, *R* reverse

and P4 + IFNα–treated groups were significantly high than P4-treated groups (Fig. 6b). As a result of RT-PCR, *TXN* was not expressed in BES in matrigel culture but was expressed only in BEE (Fig. 3g).

Discussion

In the present study, the bovine uterine glands were produced in vitro to elucidate the process of elongation of bovine embryos. In matrigel, bovine endometrial epithelial cells were cultivated and the expression of different endometrial glandular factors was investigated. Moreover, the model of progesterone and/or IFN influence on the gene expressions was also evaluated. The cells inside the living tissue usually form a three-dimensional structure with cell-cell and cell-extracellular matrix (ECM) interactions, and the cells cultured in the traditional culture system (monolayer culture) adhere to the culture plate. Monolayer cells activate excessively on proliferative signals, and cells only communicate in a two-dimensional structure (Gray et al. 2006). Cell properties are usually considered very different between in vivo tissues and monolayer cultures (Gray et al. 2006). To allow the work of cells similar to in vivo, the cells are cultivated three-dimensionally to replicate the cell-cell and cell-ECM interactions. The cells are grown by matrigel in three dimensions. Matrigel is used not only for growth but also for the analysis of cancer cell invasion and hepatocyte differentiation (Khodabandeh et al. 2017; Su et al. 2018). Matrigel, which consists mainly of laminin, type-IV collagen, and enactin, is known to be a reconstituted preparation of the basement membrane (Hughes et al. 2010). Matrigel has proved to be an effective matrix for stem cell culture because of its capacity to sustain self-renewal and pluripotency. We used a method for culturing BEE to create a gland-like structure. Matrigel was most suitable for this cultivation system at a concentration of 15% (Fig. 1). This finding reveals that matrigel contains more laminin and type-IV collagen, which can act as the glandular epithelium's basement membrane (Hughes et al. 2010). The concentration of matrigel was shown to affect morphological cell changes since aggregation levels decreased in the high matrigel concentration (over 30%). Since the expression of cell proliferation and apoptosis related genes has similarity, it was suggested that the biological activity of BEE cultured in matrigel was not different from that of monolayer cultured cells at least until 6 days of culture.

Even though the expression of *FOXA2* was not substantially different, *SERPINA14* and *GRP* expressed more frequently in BEE cultivated in matrigel than in monolayers. It was suggested that matrigel culture provide an adequate environment for *SERPINA14* and *GRP* expression like in vivo. It is generally known that FOXA2 expression is specific to glandular epithelium and not expressed in luminal epithelium. That is why the expression of *FOXA2* has considered to be due to the higher expression in monolayer cultured BEE, compared with that in the luminal epithelium. Actually, the expression of *FOXA2* was reported in rat endometrial epithelial cells cultured in vitro (Yamagami et al. 2014). The results of present study further demonstrated that *FOXA2* was expressed in BEE cultured in matrigel. Nevertheless, because the expression of *SERPINA* 14 and *GRP* was greater than those in the monolayer, BEE cultured in matrigel may be a useful approach for evaluating glandular epithelium–derived secreted factors.

In the co-cultured BEE and BES in matrigel, real-time qPCR examined the effects of 1 μM P4 on expressions of *BTC*, *PDGFB*, *PDGFA*, *MSTN*, and *IL15*. These genes have been chosen from genes that encode secretory proteins in the endometrium on the 13th day of pregnancy, which marks the start of bovine embryo elongation. Present study focussed on the cytokines and growth factors that can function on embryos in a paracrine way. Since embryo elongation is reported to be impaired in low P4 concentrations of cattle, factors related to embryo elongation may be regulated by P4. This study was given emphasis on the regulation of P4 using the martigel co-culture system on the expression of *FGF13* gene. The expression of the *FGF13* in co-culture was significantly increased by P4 (Fig. 3f). *FGF13* gene was considered to be produced by the uterine glands and to regulate the elongation of the embryo. It was reported to proliferate in skeletal muscle cells, and *FGF13* is considered to proliferate embryo trophectoderm cells during elongation (Lu et al. 2015). If the *FGF13* receptor is expressed in the embryo, the endometrium-produced *FGF13* may act on the elongation of the embryo. However, the receptor for *FGF13* has not yet been elucidated, and it will have to be revealed. In the future, it is necessary to analyze the growth factors in the protein level that act on the elongation of the embryo by using the co-culture system.

The results of the real-time qPCR analysis of endometrial tissues suggested that the expression of *SERPINA14*, a major protein of the bovine uterine fluid secreted from the glands, was regulated by P4 and IFN (Fig. 6b). However, there were no differences in the expression of *SERPINA14* in BEE which were cultured in matrigel treated without co-culture with BES. It was considered that the interaction of epithelium-stroma is important for the endometrial glandular function. It has been reported that the expression of progesterone receptor (PR) is suppressed in the endometrial epithelium at a high level of P4 (Spencer and Bazer 1995). The PR protein in the endometrial glandular epithelium on day 11 of pregnancy and in the luminal epithelium on day 13th could not be

detected (Spencer and Bazer 1995). Factors produced in stromal cells by P4 regulate the function of epithelial cells (Cunha et al. 1985). It is suggested that the culture system in which BES is present is necessary for the analysis of epithelial functions. In the present study, the expression of *SERPINA14* in matrigel cultured BEE increased in the co-culture system with BES (Fig. 6c). Stromal cells do not express *SERPINA14*. Additionally, there was no effect of P4 + IFN on the expression of *SERPINA14* when BEE were cultured without co-culture in matrigel. Therefore, it is considered that factors produced from the BES induced by P4 acted on the expression of *SERPINA14* in BEE of the co-culture and it might be the model that can reflect the physiological reaction like in vivo.

ALDOA and *TXN* are the factors that encode secretory proteins expressed in the endometrium on day13 of pregnancy (Forde et al. 2014). There was no difference in the expression of *ALDOA*; however, the expression of TXN significantly increased in IFNα + P4–treated groups compared with control ($P < 0.05$). Moreover, the expression in IFNα alone and P4 + IFNα–treated groups were significantly high than P4-treated groups (Fig. 6b). This finding was supported by Haeger et al. (2018) as they stated that bovine glandular epithelial cell line retains its epithelial phenotype in culture and forms gland acini in vitro, thereby confirming its glandular character. Cells were only reactive in low IFNα concentrations when pre-treated with steroids, thereby closely resembling implantation physiology in vivo. As well as in a correctly organized sequential sequence, E2, P4, and IFNτ act during implantation (Bartol et al. 1999). Moreover, it has been shown in previous studies that P4 is permissive for the expression of ISGs directly induced by IFNα and those induced by P4 and further stimulated by IFNα (Bazer et al. 2009; Stewart et al. 2001). As a result of RT-PCR, *TXN* was not expressed in BES in matrigel culture but was expressed only in BEE (Fig. 4).

BEE formed a gland-like structure in matrigel and further expressed specific glandular factors. Besides, BEE and BES co-cultured in matrigel were affected by P4 and IFN. It was shown that co-culture with BES is necessary for the functional analysis of BEE in vitro. Since many genes are expressed in the endometrial tissue, it is difficult to analyze only the factor produced by uterine glands. The co-culture system developed in the present study might be a useful model for the analysis of factors produced by glandular epithelium. Furthermore, by using this culture system, it becomes possible to clarify factors regulating embryo elongation and regulation of their expression. It will be able to reveal the mechanism of the embryo elongation and contribute to the improvement of the embryo transplantation technique.

Funding This work was supported by the Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (grant no. 19K22311).

Declarations

Ethical approval The animals used in this study were treated according to the guidelines for Animal Experiments in the Faculty of Agriculture of Kyushu University (no. A19-297-0) and the laws of the Japanese Government (Law no. 105 with notification no. 6).

Conflict of interest The authors declare that they have no conflict of interest.

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