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ORIGINAL ARTICLE

Establishment and characterization of immortalized bovine endometrial epithelial cells

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ABSTRACT

Bovine primary uterine endometrial epithelial cells (EECs) are not ideal for long-term studies, because primary EECs lose hormone responsiveness quickly, and/or they tend to have a short life span. The aims of this study were to establish immortalized bovine EECs and to characterize these cells following long-term cultures. Immortalized bovine EECs were established by transfecting retroviral vectors encoding human papillomavirus (HPV) *E6* and *E7*, and human telomerase reverse transcriptase (*hTERT*) genes. Established bovine immortalized EECs (imEECs) showed the same morphology as primary EECs, and could be grown without any apparent changes for over 60 passages. In addition, imEECs have maintained the features as EECs, exhibiting oxytocin (OT) and interferon tau (IFNT) responsiveness. Therefore, these imEECs, even after numbers of passages, could be used as an *in vitro* model to investigate cellular and molecular mechanisms, by which the uterine epithelium responds to IFNT stimulation, the event required for the maternal recognition of pregnancy in the bovine species.

Key words: *bovine, endometrial epithelial cell, immortalization.*

INTRODUCTION

In most mammals, conceptus implantation to the uterine endometrium consists of blastocyst hatching, migration, apposition/attachment, invasion and subsequent placental formation. It is known that close to 50% of fertilized pre-implantation embryos in mammals, including humans, fail to implant (Wilcox *et al*. 1988). The 40–50% of embryonic losses that occur between days 8 and 17 of pregnancy in cattle are thought to result from insufficient communication between the conceptus and the maternal environment (Wolf *et al*. 2003). To reduce embryonic losses in the bovine species, biochemical communication between the conceptus and endometrium needs to be characterized. However, in the bovine species continuous sampling of uterine and conceptus tissues is difficult to achieve. Therefore, development of *in vitro* models to study implantation processes is required.

Recently, we have established an *in vitro* co-culture system with bovine trophoblast CT-1 cells and primary uterine endometrial epithelial cells (EECs) that mimic the *in vivo* attachment process (Sakurai *et al*. 2012). However, primary EECs are not ideal for long-term studies, because these cells undergo some de-differentiation in culture, for example, loss of hormone and growth factor/cytokine responsiveness, and have a short life span before senescence. In addition, consistent supply of primary EECs is required, if the *in vitro* culture system is continued to be executed. In sheep, immortalized cell lines of luminal and glandular epithelial cells and stromal cells have been established and characterized (Johnson *et al*. 1999). In the bovine, a spontaneously derived bovine endometrial epithelial cell line, bovine endometrial cell (BEND), is

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used as a model to investigate the mechanisms regulating prostaglandin (PG) production (Binelli *et al*. 2001). Furthermore, bovine immortalized EECs (named bEEL) were established using lentiviral vector expressing human telomerase (Krishnaswamy *et al*. 2009). The bEEL is a good *in vitro* model to investigate the mechanism associated with the inhibition of oxytocin (OT)-induced PGF2α production by conceptus interferon tau (IFNT).

In days 8 and 17 of bovine pregnancy, during which the process of maternal recognition of pregnancy must occur (Bazer *et al*. 1991), attenuation of OT-induced PGF2α production is undoubtedly important in determining success or failure of pregnancy (Spencer *et al*. 1999). However, the subsequent events, such as conceptus attachment and limited invasion to the maternal endometrium, are also critical for the implantation processes to proceed. If the immortalized EECs, of which characteristics of primary EECs could be maintained for longer periods, were established, the *in vitro* co-culture system (Sakurai *et al*. 2012) would become a more valuable tool to study interaction as well as events associated with conceptus attachment and limited invasion to the maternal endometrium. Unfortunately, these events are often overlooked or are not able to be studied, even though early embryonic losses occur during this time period. The aims of this study were to establish immortalized bovine EECs from an early passage stage of primary EECs, and to characterize cell responsiveness to OT and/or IFNT following long-term cultures.

MATERIALS AND METHODS

Culture and immortalization of bovine EECs

Primary bovine EECs were isolated and cultured as previously described (Skarzynski *et al*. 2000). In brief, uteri of Holstein cows were obtained from a local abattoir in accordance with protocols approved by the local Institutional Animal Care and Use Committee. Healthy uteri without a visible conceptus were obtained within 10–20 min after exsanguination and immediately transported to the laboratory on ice. The stages of the estrous cycle were determined macroscopically. The cells were maintained at 37°C in air with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12, 1:1 (v:v) (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% (v/v) newborn calf serum (Invitrogen, Carlsbad, CA, USA) and antibiotic/antimycotic solution (Invitrogen).

Retroviral vectors were kindly provided by Dr. T. Kiyono, National Cancer Center Research Institute, Tokyo, Japan. Production of recombinant retroviruses was performed according to the methods described by Kyo *et al*. (2003). Briefly, retroviral vectors, human papillomavirus (HPV)-16 *E6*, HPV-16 *E7*, human telomerase reverse transcriptase (*hTERT*) and packaging construct, pCL-10A1 (IMGENEX., San Diego, CA, USA), were co-transfected into Platinum-GP (Plat-GP) cells (Cell Biolabs, Inc., San Diego, CA, USA) using FuGene6 (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. The culture fluid was harvested 48 h after transfection, and viral supernatant was collected through the filtration with a 0.45-μm low protein binding filter (Millipore, Bedford, MA, USA). Viral supernatant was then combined with 1 mL of serum-free DMEM/Ham's F-12 medium containing protamine sulfate (Sigma-Aldrich) and overlaid onto third-passage primary EECs that had been grown to 70% confluence in six-well dishes.

Cell proliferation assays

Cell proliferation assays were conducted using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. Primary or immortalized EECs were seeded onto a six-well plate coated with or without collagen type IA and cultured as described above. After attachment (0, 24 or 48 h), seeded cells were treated with 1 mL of DMEM/Ham's F-12 with CCK-8 reagent (1: 10, v/v), and the cells were incubated for 1 h. To estimate proliferated cell numbers, absorbance was measured for each well at a wavelength of 450 nm using an auto-microplate reader (Wallac 1420; Perkin Elmer Japan, Tokyo, Japan).

RNA extraction and analysis

Total RNA was extracted from EECs with ISOGEN (Nippon Gene, Tokyo, Japan) according to the protocol provided by the manufacturer. For PCR, isolated RNA (total 1 μg) was reverse-transcribed to complementary DNA (cDNA) using ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) including 1 × RT buffer, enzyme mix, and primer mix in a 10 μL reaction volume, and the resulting cDNA (RT template) was stored at 4°C until use. The cDNA reaction mixture was diluted 1:10 using deoxyribonuclease (DNase)- and ribonuclease (RNase)-free molecular biology-grade water and 3 μL were taken for each amplification reaction. PCR was carried out with 0.5 units of ExTaq HS polymerase (Takara Bio, Tokyo, Japan), 1 × ExTaq HS buffer, 0.2 μmol/L of the oligonucleotide primers described in Table 1 and 0.2 mmol/L of dNTP in a final volume 20 μL. The thermal profile for PCR was at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. The PCR products were separated on a 1.5% agarose gel containing ethidium bromide and were visualized under UV light.

Measurement of PGF2α

To evaluate the OT responsiveness, primary and immortalized EECs that had been cultured for 48 h were treated with a range (1 to 1000 ng/mL) of OT (Teikoku Hormone MFG Co., Tokyo, Japan) for 24 h. After incubation, the conditioned media were collected, from which PGF2α concentrations were determined by enzyme immunoassay (EIA) as previously described (Woclawek-Potocka *et al*. 2004). The remaining cells were subjected to cell number analysis using the CCK-8 method. The final PGF2α concentrations were obtained following the cell number correction from the value of CCK-8.

Immunocytochemistry

EECs were plated onto non-coated six-well dishes and analyzed for expression of epithelial-specific marker cytokeratin or stromal-specific marker vimentin. The cells were grown to 70% confluence and fixed in 4% paraformaldehyde at room temperature for 15 min. Non-specific binding was inhibited by blocking the cells with 10% normal

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Table 1 Primers for RT-PCR analyses

Name (GenBank accession no.)	Sequence	Length (bp)
E6	F: GCAACAGTTACTGCGACGTG	
(FJ237042.1)	R: GGACACAGTGGCTTTTGACA	234
E7	F: CATGCATGGAGATACACCTACAT	
(FJ237041.1)	R: TTATGGTTTCTGAGAACAGAT	298
hTERT	F: CGGAAGAGTGTCTGGAGCAA	
(BC172541.1)	R: GGATGAAGCGGAGTCTGGA	145
IRF1	F: GCTGGGACATCAACAAGGAT	
(XM_001787207.1)	R: CTGCTCTGGTCCTTCACCTC	163
IRF2	F: AAACTGGGCCATCCATACAG	
(XM_592435.3)	R: TTAGAAGGCCGCTCAGACAT	192
IRF9	F: GAGAACTGCTGTCCCTGGAG	
(NM_001024506.1)	R: GGGTTTGGGAAAGATCACCT	179
MX1	F: GTCCCTGCTAACGTGGACAT	
(NM_173940)	R: ACCAGGTTTCTCACCACGTC	155
MX2	F: GCAGATCAAGGCACTCATCA	
(NM_173941.2)	R: ACCAGGTCTGGTTTGGTCAG	168
STAT1	F: GCCTCAAGTTTGCCAGTGGC	
(BC151378.1)	R: GGCTCCCTTGATAGAACTGT	314
STAT2	F: CAGCCAGTACCAGATGCTCA	
(XM_588270)	R: CCAGCAGTGGCTCTAAGTCC	296
ACTB	F: CTCTTCCAGCCTTCCTTCCT	
(BC102948)	R: GGGCAGTGATCTCTTTCTGC	178

F, forward; R, reverse.

goat serum (Invitrogen) for 30 min. The cells were incubated with primary antibodies, cytokeratin (Dako, Glostrup, Denmark; diluted 1:500 in phosphate-buffered saline (PBS)) or vimentin (Dako, diluted 1:50 in PBS), for 2 h, and with biotin-conjugated secondary antibody for 30 min. Cells were then incubated with Avidin Alexa 568 Streptavidin Conjugates (Invitrogen) for 30 min. The cells were then examined under light microscope (BX-51; Olympus, Tokyo, Japan).

Statistical analysis

The concentrations of PGF2α in the culture media of OT-treated primary and immortalized EECs, measured by the enzyme immunoassay, represent the results of triplicate samples within an assay, and were expressed as the mean ± SEM. These data were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's test for multiple comparisons with the StatView statistical analysis software (version 5; SAS Institute Inc., Cary, NC, USA). Differences of $P < 0.05$ were considered to be significant.

RESULTS

Establishment and characterization of immortalized EECs

The primary bovine EECs were obtained as previously described (Skarzynski *et al*. 2000). These cells proliferated for a limited number of passages. To extend the life span of EECs, attempts were made to establish immortalized EECs through the transfection of *E6*, *E7* and *hTERT* genes into primary EECs. Most transfected EECs exhibited various degree of transfected gene expression, inconsistent growth rates or OT responsiveness. However, one line named imEECs exhibited the similar expression of three transfected genes, of which characteristics were subsequently analyzed. Under the microscope, the slight morphological changes in imEECs were observed. Soon after initial plating, imEECs were generally flat to cuboidal in shape, namely 'cobblestone' appearance (Fig. 1A). As the cells grew to confluence, these cells appeared as epithelial whorls, characteristics of the primary EECs. Expression of *E6*, *E7* and *hTERT*, examined by RT-PCR, showed the expression of *E6*, *E7* and *hTERT* messenger RNAs (mRNAs) in a similar manner (Fig. 1B). More importantly, transcripts of ovarian steroid hormone receptors (*ER* and *PR*) and type I interferon receptors (*IFNAR1* and *2*) were also found in imEECs even after 60 passages (Fig. 1C).

Proliferation of primary and immortalized EECs

ImEECs were grown without apparent signs of senescence for at least 60 passages. Primary EECs at third passages and imEECs at 60th passages were plated onto collagen-coated or non-coated plates and cultured for 24–48 h. Proliferation of primary and immortalized EECs was assessed with the CCK-8 method. As shown in Figure 2, imEECs maintained the proliferation ability equivalent to primary ECCs even after 60 passages (0 h in the figure). Moreover, differences in imEECs' proliferation were seen after 24 h culture and further increase in proliferation was observed at 48 h.

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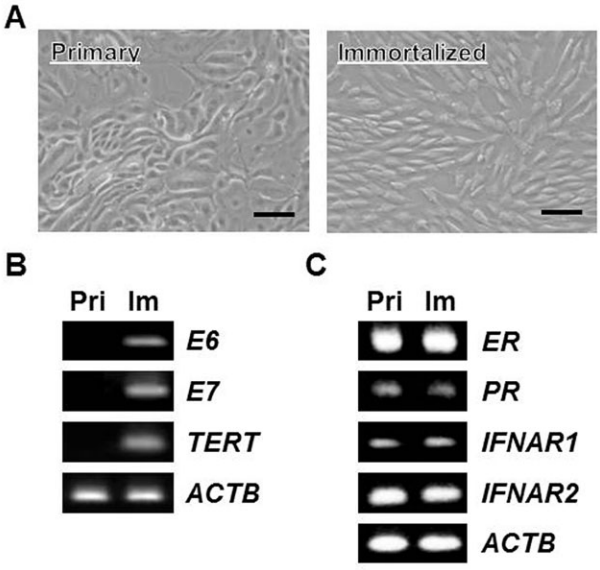


Figure 1 The morphology and characterization of immortalized bovine uterine endometrial epithelial cells (EECs). (A) Immortalized bovine uterine EECs were obtained by transfection of retroviral vectors, containing HPV-16 *E6*, *E7* and *hTERT* genes to extend their life span. The representative photographs of EECs are shown (left: primary, right: immortalized). Bar = 200 μm. (B) The expression of HPV-16 *E6*, *E7* and *hTERT* mRNAs in transfected EECs. Transcripts were examined by RT-PCR in primary and immortalized EECs. *ACTB* was used as an internal control. (C) The expression of steroid and interferon A (IFNA) receptor gene transcripts in primary and immortalized EECs. RNAs, extracted from primary or immortalized EECs at 60th passage, were subjected to PCR analysis. *ACTB* was used as an internal control.

Effect of OT or IFNT treatment on primary and immortalized EECs

Effects of OT or IFNT treatment were tested for primary and immortalized EECs. OT treatment induced a concentration-dependent increase in PGF2α accumulation in both cells (Fig. 3A). More importantly, IFNT treatment induced the expression of IFN stimulated gene transcripts; interferon-inducible Mx proteins (*Mx1* and *Mx2*), interferon regulatory factors (*IRFs*), and signal transducers and activator of transcriptions (*STATs*). Thus, imEECs have maintained the ability to respond to OT and IFNT stimulation even after 60 passages and the degree of response at this stage was similar to those of primary EECs (Fig. 3).

Expression of vimentin and cytokeratin in immortalized EECs

Immunofluorescence studies were then performed. Primary EECs exhibited positive signals for cytokeratin and negative for vimentin, while imEECs cells stained positive for both cytokeratin and vimentin (Fig. 4).

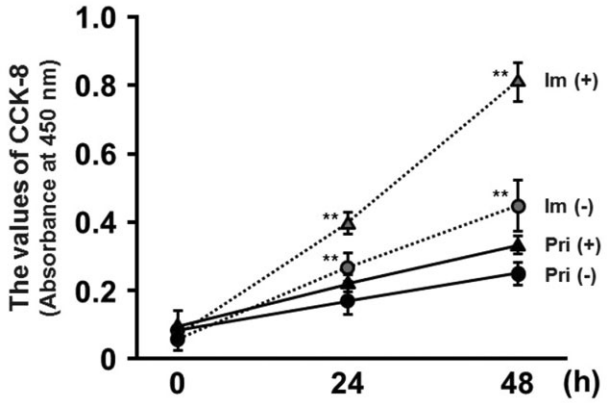


Figure 2 Proliferation of primary and immortalized endometrial epithelial cells (EECs). Primary (Pri) and immortalized at 60th passage (Im, broken line) EECs were cultured on collagen type IA-coated (+, triangle) or non-coated (–, circle) six-well plates for up to 48 h. The values of CCK-8 (MTT absorbance at 450 nm) were measured at 450 nm at 0, 24, or 48 h ($n = 3$). Duplicate samples were examined for each treatment, and three independent experiments were performed. Values represent means ± SEM. Statistical significance: $^{**}P < 0.01$ vs. Pri (–) within a time period.

DISCUSSION

Early embryonic loss is undoubtedly one of the major causes for not being able to improve pregnancy rates, particularly multiparous cows in Japan and throughout the world. It has been recognized numerous times that biochemical communications between conceptus and uterine endometrium during the peri-implantation period must be elucidated, if the pregnancy rate could be improved. However, a lack of experimental systems in studying such communication has limited investigators to elucidate the conceptus-endometrial dialogue. Stable EECs, which could overcome such limitations, are ideal to study trophoblast-endometrial epithelial cell interactions. Here, we established immortalized bovine EECs (imEECs), which have been maintained without any apparent morphological change in continuous cultures for more than 60 passages (Figs 1,2).

The established imEECs responded to OT treatment, resulting in a concentration-dependent PGF2α production (Fig. 3A). These cells also responded to IFNT treatment. These results show that established imEECs maintained the characteristics of uterine EECs during long-term culture periods. Thus, the imEECs could be a useful experimental model for studying biochemical-molecular communication between the conceptus and uterine epithelium, the events prerequisite for the implantation process to proceed. Since we established an *in vitro* co-culture system with bovine trophoblast CT-1 cells and EECs that mimic the *in vivo* attachment process (Sakurai *et al*. 2012), a next step forward is to

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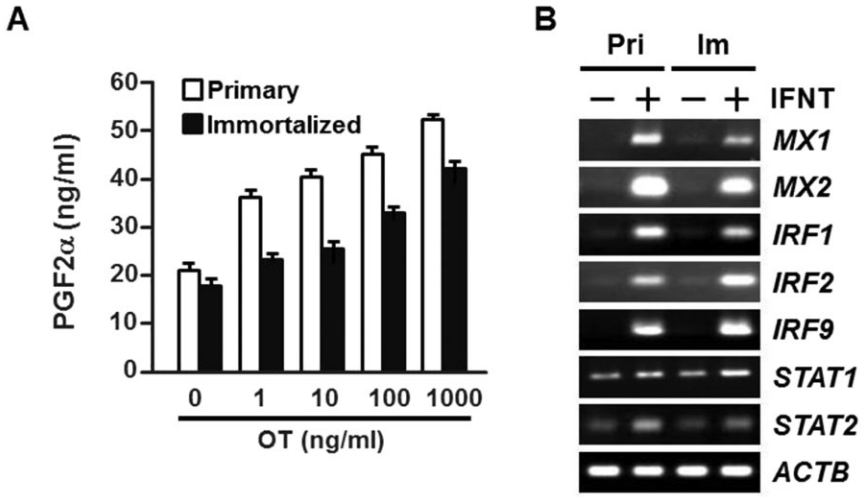


Figure 3 Effect of OT on PGF2α accumulation and the expression of interferon (IFN) stimulated genes following recombinant ovine IFNT treatment in immortalized endometrial epithelial cells (imEECs). (A) The primary (white bar) and immortalized at 60th passage (black bar) EECs were cultured on 48-well plates coated with collagen type IA. The cells were treated with increasing concentrations of OT (0, 1, 10, 100 or 1000 ng/mL) and PGF2α accumulation in conditioned media after 24 h incubation were measured using an enzyme immunoassay. The concentrations were corrected for cell numbers as determined by Cell Counting Kit-8 (CCK-8) (see Materials and Methods). The mean concentration of prostaglandin F2α (PGF2α) was derived from an average of three wells within a single plate experiment. Values represent means ± SEM. (B) The expression of IFN-stimulated gene transcripts in primary and immortalized EECs. The primary (Pri) and immortalized (Im) EECs were seeded on six-well plates coated with collagen type IA. The cells were treated with IFNT (1 μg/mL) for 24 h and the expression of IFN stimulated gene transcripts was analyzed by RT-PCR. IFNT stimulated genes include interferon-inducible Mx proteins (*Mx1* and *Mx2*), interferon regulatory factors (*IRF1*, *2* and *9*) and signal transducers and activator of transcriptions (*STAT1* and *2*). *ACTB* was used as an internal control.

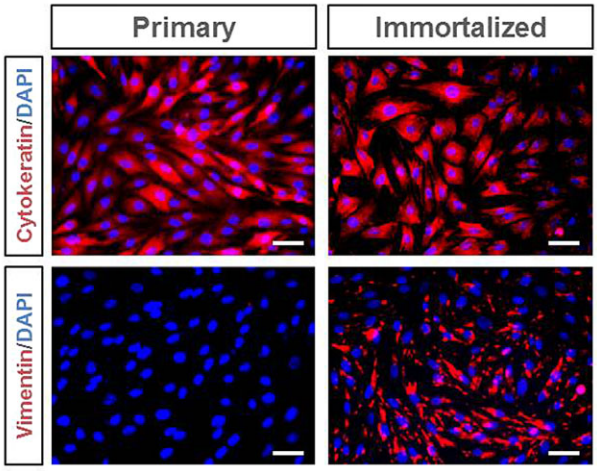


Figure 4 Immunofluorescence characterization of the immortalized endometrial epithelial cell (imEECs). Immunofluorescence staining analysis on primary (left panels) or immortalized at 60th passage (right panels) EECs was carried out using antibodies against cytokeratin (upper panels) as epithelial marker, or vimentin (lower panels) as stromal marker. Bar = 200 μm.

examine if the imEECs could be used to mimic trophoblast CT-1 cell attachment processes, similar to those seen when primary EECs were used in our co-culture system.

Although the established imEECs maintained the characteristics of uterine EECs following numerous passages, these cells were positive for both epithelial marker, cytokeratin and stromal marker, vimentin. It has been reported numerous times that cytokeratin-positive epithelial cells acquire vimentin during *in vitro* culture periods (Bergh *et al*. 1984; Wang *et al*. 2000; Zeiler *et al*. 2007). The co-expression of both epithelial and mesenchymal markers *in vitro* has been explained by a limited epithelial to mesenchymal transition (EMT) (Pagan *et al*. 1996). It was also thought that this transition correlates with a loss of cell-to-cell contacts that result from cell disaggregation during the period of epithelial cell culture preparation (Zeiler *et al*. 2007). *In vivo*, the expression of vimentin, while trophoblasts maintained epithelial nature, has recently been reported (Yamakoshi *et al*. 2012). However, this was at the trophoblast side, not at the uterine epithelium, which was predicted two decades ago (Denker 1993). In addition to these observations, a previous study (Krishnaswamy *et al*. 2009) also indicated that the development of conceptus and endometrium requires biochemical communications as well as cell-to-cell interactions, if a pregnancy is to proceed, and suggest that the communication between the conceptus and uterine epithelium must be studied in not only co-culture systems but also those in flexible

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and dynamic cell environments. For these reasons, imEECs could be a more valuable tool than primary EECs in studying such changes as well as gene expression as the implantation process proceeds.

Conclusion

Our established imEECs, even after a number of passages, offer an *in vitro* model to elucidate molecular mechanisms associated with proper conceptus-endometrial dialogue in the bovine species, in which *in vivo* experiments are difficult to conduct.

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