Establishment and characterization of immortalized bovine endometrial epithelial cells

Hanako BAI,1,† Toshihiro SAKURAI,1,‡ Rulan BAI,1 Sachiko YAMAKOSHI,1 Etsunari AOKI,1 Mariko KUSE,2 Kiyoshi OKUDA2 and Kazuhiko IMAKAWA1

1Laboratory of Theriogenology and Animal Breeding, Graduate School of Agricultural and Life Science, the University of Tokyo, Tokyo, and 2Laboratory of Reproductive Physiology, Graduate School of Environmental and Life Science, Okayama University, Okayama, Japan

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

Correspondence: Kazuhiko Imakawa, Laboratory of Theriogenology, Veterinary Medical Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan. (Email: akaz@mail.ecc.u-tokyo.ac.jp) Present addresses: †Mito Research Center, Meiji feed Co., Ltd, Ibaraki, Ibaraki 311-3123, Japan. ‡Laboratory of Radiophysics, Department of Life Science, Yokohama College of Pharmacy, Kanagawa 245-0066, Japan. Received 19 June 2013; accepted for publication 27 January 2014.

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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 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. 2008). 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% CO2 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 I 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 MPG 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
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 EECs 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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**Table 1 Primers for RT-PCR analyses**

| Name (GenBank accession no.) | Sequence | Length (bp) |
|-----------------------------|----------|-------------|
| E6 (FJ237042.1)             | F: GCAACAGTTACTGCGACGTG | 234         |
| E7 (FJ237041.1)             | R: GGACACAGTGCGTTTTGACA | 298         |
| hTERT (BC172541.1)          | F: CATHGATGGAGATACACCTACAT | 145         |
| IRF1 (XM_001787207.1)       | R: GGATGAAAGCGAGCTGTTGA | 163         |
| IRF2 (XM_592435.3)          | F: AACTGGGAGATCAAAAGGA | 192         |
| IRF9 (NM_001024506.1)       | R: GGTTTGGGAAAGATCAACTC | 179         |
| MX1 (NM_173940)             | F: GTCCCTGCTAAGCTGGACAT | 155         |
| MX2 (NM_173941.2)           | R: ACCAGTTCACCCAGTCGTC | 168         |
| STAT1 (BC151378.1)          | F: ACCAGGTCTGGTGTCTGAG | 314         |
| STAT2 (XM_588270)           | R: GGCTCCTCTGTAAGAAGCTG | 296         |
| ACTB (BC102948)             | F: CTCCTCCAGCCTTCTCCTCCT | 178         |

F, forward; R, reverse.
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).

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
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
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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