

Establishment and characterization of porcine mammary gland epithelial cell line using three dimensional culture system

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3차원 배양 시스템을 이용한 돼지 유선 상피 세포 주 특성과 설정

정학재

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Abstract To study and validate tissue-specific promoters and vectors, it is important to develop cell culture systems that retain the tissue and species specificity. Such systems are attractive alternatives to transgenic animal models. This study established a line of porcine mammary gland epithelial cells (PMECs) from a primary culture based on the cellular morphology and mRNA levels of porcine beta-casein (CSN2). The selected PMECs were stained with the cytokeratin antibody, and were shown to express milk protein genes (CSN2, lactoferrin, and whey acidic protein). In addition, to confirm the acini structure of PMEC932-7 in 3D culture, live cells were stained with SYTO-13 dye, which binds to nucleic acid. The acini of these PMECs on matrigel were formed by the aggregation of peripheral cells and featured a hollow lumen. The system was demonstrated by testing the effects of the culture conditions to cell culture including cell density and matrigel methods of the PMECs. These results suggest that PMECs possess the genetic and structural features of mammary epithelial cells.

요약 조직특이적 프로모터 및 벡터를 연구하고 검증하기 위해서는 조직 및 종의 특이성을 유지하는 세포 시스템을 개발하는 것이 바람직하다. 이러한 시스템은 형질전환동물 모델에 대한 효과적인 대안이다. 우리는 베타 카제인 (CSN2)의 세포 형태와 mRNA 수준에 기초하여 일차 배양으로부터 돼지 유선 상피 세포 주 (PMECs)를 확립하였다. 선택된 PMECs는 cytokeratin 항체에 의해 염색되었으며, 유선 상피 세포에 존재한다고 생각되어지는 유즙 단백질 유전자 (CSN2, 락토페린 및 유청 단백질)를 발현하는 것으로 나타났다. 또한, 3D 배양에서 PMECs932-7의 acini 구조를 확인하기 위해 살아있는 세포를 핵산에 결합하는 SYTO-13으로 염색하였다. 우리는 매트릭겔 (matrigel)에 있는 PMECs의 acini가 말초 세포의 응집에 의해 형성되고 공간의 lumen을 특징으로 한다는 것이 관찰하였다. 우리는 PMECs의 matrigel 사용법과 세포 밀도를 포함한 세포 배양 조건의 영향을 시험함으로써 시스템을 시연했다. 이러한 결과는 PMECs의 유선 상피 세포는 유전적 또는 구조적 특징을 갖고 있음을 시사하고 있다.

Keywords : Acinus; 3D culture; Matrigel; Porcine beta-casein; Porcine mammary gland epithelial cell

1. Introduction

protein in bioreactors using lactating transgenic animal system is the most popular strategy [1]. An advantage

Production of valuable therapeutic recombinant

of this system is that transgenic protein expression is

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often 10 - 100-fold greater than is attainable from mammalian cell culture systems. Transgenic animal bioreactors produce proteins with suitable structures, stability, bioactivity and safety [2]. Several animal models are available for transgenic protein production with differing levels of suitability. Bioreactors system using mice and rabbits offer several advantages such as high fecundity and rapid generation time, but quantity of milk produced in these animals is limited. However, pigs offer a relatively high milk production capacity (up to 400 L/year), short gestation period, large litter size, and relatively small breeding area requirement, compared with animals such as dairy cattle [3-5]. A number of transgenic studies using mammary gland-specific promoters have been reported [6-8].

During the female reproductive cycle, the mammary gland recapitulates development (e.g., cell proliferation and organization) and subsequent involution (e.g., apoptosis of certain cells) observed during puberty, pregnancy and parturition [9]. Branching morphogenesis of mammary glands is regulated by extracellular matrix (ECM) and ECM-receptors, and is characterized by epithelial invasion of adipose tissue [10].

Whereas mammary epithelial cell lines grown on collagen gels have been studied with respect to tissue-specific function and phenotype, *in vitro* culture conditions have not generated the polarized structure of mammary glands observed *in vivo* [11]. Monolayer and two-dimensional (2D) culture are less useful for expression of mammary gland-specific proteins [12]. It is more favorable to employ a three-dimensional (3D) culture system using Matrigel to provide a microenvironment that is physiologically similar to *in vivo* conditions [13]. Several studies have reported the establishment of 3D porcine mammary gland epithelial cell (PMEC) cultures from the mammary glands of non-parous female pigs (gilts); [14]. Mammary gland-specific genes such as lactoferrin (LTF), beta-casein (CSN2), and beta-lactoglobulin (BLG), were shown to be expressed in PMECs following induction by lactogenic hormones. PMECs isolated

from lactating sows have been employed for measuring transfection efficiency using GFP-encoding genes [15]. Spontaneously immortalized-PMECs have been maintained for greater than 70 passages, retaining epithelial lineage and capability of forming functional structures in Matrigel [16]. However, unlike mouse mammary epithelial cells cultured on Matrigel, acini and luminal structures have not been definitively reported for PMECs.

Development of a tissue-specific expression vector is critical for production of transgenic animal platform for biopharmaceutical production. Particularly, *in vivo* validation of developed vectors containing temporal/spatial specific promoter sequences is expensive and time consuming process. Thus, *in vitro* validation using expression system of cell lines similar to that of *in vivo* tissue would be time-saving and cost-effective way before apply for animals. In this study, we aimed to establish a mammary gland cell line system to verify constructed vectors that will be inserted into porcine genome for production of recombinant proteins in porcine milk.

2. Materials and Methods

2.1 Primary culture and colony selection

To obtain PMECs, 1 g of mammary gland tissue was surgically obtained by excisional biopsy from Landrace gilts during the lactation period (5 weeks post parturition). The tissue was washed 5 times with 1× D-PBS (Dulbecco's phosphate-buffered saline; Invitrogen, Carlsbad, CA, USA) and once with growth media: Dulbecco's modified Eagle's medium (DMEM)/F-12 (Invitrogen) supplemented with 10% FBS (heat-inactivated fetal bovine serum; Invitrogen), 5 µg/mL insulin (Sigma, St. Louis, MO, USA), 10 ng/mL EGF (mouse epidermal growth factor; Sigma), 50 units/mL penicillin and 50 µg/mL streptomycin (Invitrogen). The tissue was cultured for one week in 75 cm² plastic tissue culture flasks and subcultured

using 8 μm cell strainers (Sigma), (ratio of 1:2 at 3 days intervals). PMECs were grown in growth media and incubated at 38.5°C in a humidified atmosphere of 5% CO_2 . After 50 passages through the subculturing system, 10 clones with high viability were selected using a colony cylinder (Sigma). Clones with high CSN2 expression were seeded into 96-well plate at a concentration of 1 cell/well and cultured for 3 days. Colonies with high proliferation were chosen for transfer to 24-well plates. Cells were then transferred to a 25 cm^2 flask and subsequently to a 75 cm^2 flask. This process was repeated four times. Finally, cells in the 25 cm^2 flask from the fourth repetition (named PMEC932-7) were examined for porcine CSN2 mRNA expression levels using quantitative real-time PCR.

2.2 Three-dimensional culture and cell staining

Three-dimensional (3D) cultures were generated using the Matrigel Matrix (BD Biosciences, Hampton, NH, USA) following the modified instruction manual. Matrigel was thawed at 4°C for at least for 16 h. A confocal dish (SPL) was coated with a mixture of serum-free DMEM/F-12 medium and matrigel at equal volumes, and incubated at 38.5°C for 30 min. PMECs were then seeded at a density of 2.5×10^4 cells/mL. Growth media was changed every 3 days. After the media was aspirated, cell masses were incubated for 10 min at room temperature with SYTO-13 dye (5 μM ; Molecular Probes, Inc., Eugene, OR, USA) in growth media and were washed with PBS (phosphate-buffered saline; Invitrogen). A section of Matrigel was prepared on a slide glass with mounting solution. Fluorescence was visualized using the FV300 scanning laser confocal microscope (FV-300, Olympus, Tokyo).

2.3 Hematoxylin and eosin staining

Mammary gland tissue samples were collected from Landrace pigs lactating and dry Landrace pigs, respectively. The samples were fixed in Bouin's solution (75% picric acid, 10% formaldehyde, 5%

glacial acetic acid), dehydrated through an ethanol series (70 - 100%), cleared in xylene (Sigma), and embedded in paraffin. All paraffin blocks were cut to a thickness of 4 μm and stained with Harris hematoxylin solution (Sigma) and eosin (Fluka Biochemika, Buchs, Switzerland). These samples were observed by light microscopy.

2.4 RNA isolation

Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA was removed by on-column DNase I digestion (RNase-Free DNase set; Qiagen).

The total RNA concentration was determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

2.5 RT-PCR and quantitative real-time PCR

cDNA was synthesized from 800 ng (20 μL reaction) of total RNA using the 1st Strand cDNA Synthesis kit for RT-PCR (Roche Diagnostics, Mannheim, Germany). Quantitative real-time PCR was performed using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) reaction mixture with 2 μL of cDNA as template, 4 mM of MgCl_2 , 1 μM primer mixture, and 0.2 μM probe mixture. The thermal profile was as follows: pre-incubation at 95°C for 10 min, then 45-cycle amplification at 95°C for 5 s, 68°C for 5 s, and 72°C for 9 s. Melting was conducted from 45°C to 95°C at an increment of 0.5°C/s and cooling was conducted at 40°C for 30 s. The names of the genes evaluated and the primer sequences used are given in Table 1. RT-PCR was performed using the Light Cycler FastStart DNA Master SYBR Green I (Roche Diagnostics) reaction mix with 2 μL cDNA and 1 μM of each primer. This step involved a denaturation program (95°C for 10 min) followed by an amplification and quantification program repeated 45 times (95°C for 10 s, 58°C - 63°C for 5 s, 72°C for 8 s with a single fluorescence

Table 1. A list of 4 genes, along with their GenBank accession No, and the DNA primer sequences used for RT-PCR and quantitative real-time PCR analyses.

Genes	GenBank Accession No	Product size	Primers
CSN2	NM_214434	210 bp	F ATC CTT ACA CCG AGC CCA TC R GTT AGG CTC TGG CCT TCC AC
LTF	M81327	328 bp	F GTG CCC AAC AGT AAT GAG AGA TAC TA R AGT CTT TTC CGT ATC TTC CAA ACT GAG
WAP (whey acidic protein)	NM_213841	241 bp	F CTC TGC TTC GAG AAA AAT GAG TG R ATG GTT TTT ATT AGC AGC AGA TAG GAG
GAPDH	AF017079	159 bp	F GGC CTC TCT CCT CCT CGC R ATT GCC CTC AAC GAC CAC

measurement), and finally a cooling step at 40°C for 30 s. GAPDH was used as a control to normalize the results. The PCR products were identified by gel electrophoresis on a 2% agarose gel stained with ethidium bromide.

2.6 Immunocytochemistry

PMECs were grown to less than 60% confluence on 18 mm round cover glasses. The medium was removed and washed with 1× PBS. Cells were then fixed for 10 min using 4% paraformaldehyde (Sigma) and were treated with 0.3% Triton X-100 (Bio-Rad, Hercules, CA, USA) for 20 min, followed by blocking solution (2% bovine serum albumin in PBS) for 60 min. Cells were subsequently incubated with primary antibody (monoclonal mouse anti-human cytokeratin clone: AE1/AE3; DAKO) for 60 min, washed three times with PBS, and incubated with secondary antibody (Anti-Mouse IgG-FITC; Sigma) for 60 min. Phase-contrast and fluorescence microscopy was performed with the FV300 scanning laser confocal microscope (Olympus Optical, Tokyo, Japan).

2.7 Statistical analysis

Real-time RT-PCR data were analyzed by one-way analysis of variance using SPSS statistical package ver. 21.0 for Windows. A t-test was performed for

comparisons between each groups. All data are expressed as the mean ± standard deviation. The hypothesis was rejected when probability was p value < 0.05.

3. Results

3.1 Lactating mammary gland and dome formation of porcine mammary gland cells

To investigate the internal structure of the mammary gland, we stained porcine mammary gland tissues (PMGT) with hematoxylin and eosin (H&E). We found that well-developed luminal structures surrounded by epithelial cells were present in the H&E stained sections of lactating PMGT (Fig. 1A), but the internal structure were only poorly developed in the dry PMGT (Fig. 1B). As seen in mouse mammary morphogenesis [10], we observed formation of cell masses with a fat pad with a reticular structure in the lactating PMGT. In our porcine mammary gland model, after cell masses were grown on collagen-coated dishes for 5 days we also observed blister-like structures that became dome-like (Fig. 1C), similar to the structures formed by COMMA-1D cells and primary mouse mammary epithelial cells in culture [17, 20, 21].

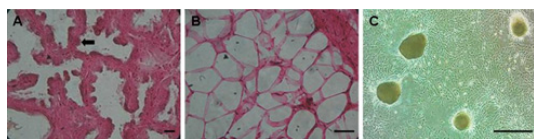


Fig. 1. Morphological difference of PMGT and dome formation of PMEC. (A, B) Comparison between the lactation (A) and dry periods (B) by hematoxylin and eosin staining of 4- μ m sections prepared from porcine mammary gland tissue. It showed internal structure of mammary gland. (C) Morphology of PMEC on coated collagen. After five days, dome formation appeared on collagen-coated dish. Scale bars, 200 μ m.

3.2 Selection and characteristics of PMEC932-7

Morphologically, cells in the first passages of the culture were heterogeneous, including fibroblast-like cells and epithelial cells. After 50 passages through the subculture/selection system, the PMEC932 cell clone was selected by its epithelial-like cell type with cobblestone morphology. To select the most suitable

cell line clone as in vitro model for mammary gland tissue, we quantified mRNA expression levels of a mammary gland-specific gene, CSN2 in the PMEC932 cell clones. This clone showed an eight-fold higher expression of porcine CSN2 than that of other PMEC932 cell clones (Fig. 2A). To characterize epithelial feature, we performed immunocytochemistry in cells from different passages of PMEC932 cell line by using an epithelial cell-specific cytokeratin antibody. We observed that unselected PMECs (early passages) were mixed with epithelial cells and other cell types (Fig. 2B-a, b) while an epithelial cell marker, cytokeratin was presented in the selected PMEC (Fig. 2B-c, d). Expression levels of the milk protein genes CSN2, lactoferrin (LTF), and whey acidic protein (WAP) were compared between selected PMEC932 cells cultured 3D culture system and PMGT. The relative amounts of expressed CSN2, LTF, and WAP mRNA in PMEC932-7 were not different between two groups (Fig. 2C).

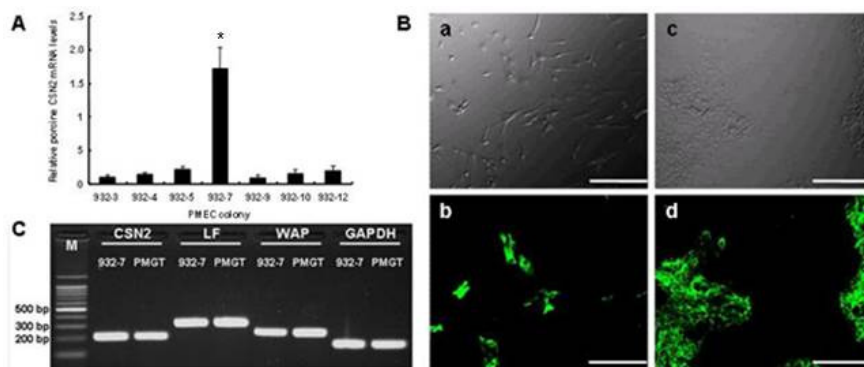


Fig. 2. Selection and verification of PMEC932-7. (A) Expression patterns of porcine CSN2 gene in PMEC932 clones. Real-time RT-PCR showed the porcine CSN2 gene expression level in PMEC932 clones. (B) Fluorescence microscopic analysis of early passage PMEC and PMEC932-7 cells stained with cytokeratin antibody. a, transmission image of PMEC; b, fluorescence image of PMEC; c, transmission image of PMEC932-7; d, fluorescence image of PMEC932-7. Scale bars, 50 μ m. (C) Expression of milk protein genes in PMEC932-7 versus PMGT. PMEC932-7 RNA isolated from cultured cells on a Matrigel-coated dish. M, DNA ladder marker; 932-7, PMEC932-7; PMGT, porcine mammary gland tissue; CSN2, Porcine beta-casein; LF, porcine lactoferrin; WAP, porcine whey acidic protein; GAPDH, porcine glyceraldehyde-3-phosphate dehydrogenase.

3.3 Acinar development in 3D culture of PMEC932-7 cells

PMEC932-7 cells were seeded on a Matrigel-coated dish. After a cell mass was observed at 4 h, cells were monitored with a 1 h interval during the following 16 h. PMEC peripheral cells aggregated to form acini (Fig. 3).

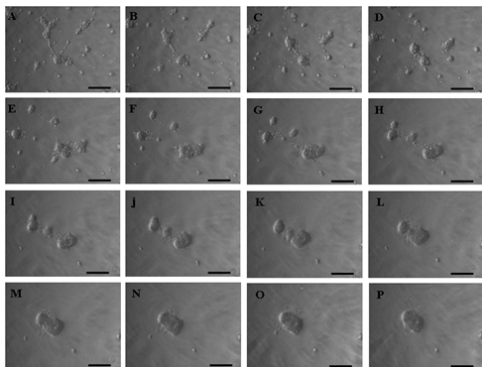


Fig. 3. Formation process of acini structures at 3D culture. PMEC932-7 cells were cultured on Matrigel for 4 h and observed under cultured cell monitoring system CCM-500F (ASTEC, Fukuoka, Japan). A~P, 1-h interval; Scale bars, 500 μ m.

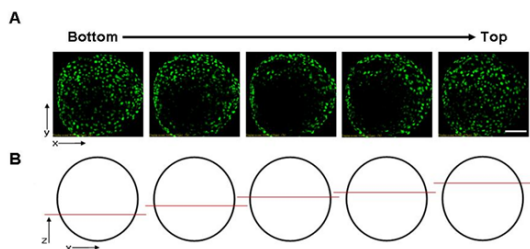


Fig. 4. Lumen structure of the PMEC932-7 at 3D culture. For generation of lumen structure, PMEC932-7 was cultured for 15 days. (A) Transmission image is showed a simple spherical form. (B) The schematic diagrams illustrate the position of the optical section. Serial confocal cross sections (stained with SYTO-13) showed a hollow lumen structure. Scale bars, 100 μ m.

These acini resembled the small units of alveolar structures seen during mammary gland development. To confirm the acini structure of PMEC932-7 in 3D

culture, live cells were stained with SYTO-13 dye, which binds to nucleic acid. We observed that polarized cells and a hollow lumen in the PMEC932-7 cell masses after 15 days in 3D culture (Fig. 4), indicating that PMEC932-7 cells formed acini structures that possessed important characteristics of glandular epithelia *in vivo*.

4. Discussion

Unlike most mammalian organs, the mammary gland recapitulates certain developmental steps (e.g., cell proliferation and involution) observed during female puberty, pregnancy and parturition [12]. For validation of vectors that expressed transgenes in milk-producing animals, mammary gland cell lines should have unique mammary epithelial cell characteristics such as differentiation and degeneration by inductive signals.

Blister/dome-like structures formation with a fat pad with a reticular structure in the PMEC932 are in line with previous finding that mouse mammary morphogenesis involves mammary gland cell invasion of adipose tissue in response to a lactation signal [10], because domes were generated by focal accumulation of fluid, suggesting the development of a polarized phenotype, and implying the capacity for transport functions [17].

In addition, selection of a clone from PMEC932 cell line by quantifying expression levels of mammary gland specific gene, CSN2 is a rapid and reliable experimental procedure because comparable levels of tissue specific genes such as LTF and WAP were expressed, suggesting that the selected PMEC932-7 cells possess intrinsic characteristics of mammary epithelial cells in terms of gene expression.

Formation of acini structures in PMEC932-7 cells suggested the cells potentially very similar to *in vivo* glandular epithelia because acini structure is a characteristic feature of mammary gland epithelial cells cultured on Matrigel [18] and lumen is formed by

apoptosis of the internal cells in primary mouse mammary epithelial cells and tube elongation and branching, resulting in tubular networks after acinus formation [16]. Interestingly, the acini formation was collapsed with defective lumen formation [19]. We also observed that acini were not formed without lactation hormones (data not shown).

In conclusion, to the best of our knowledge, this is the first study to observe formation of acini in PMEC cells on Matrigel occurring as a result of peripheral cell aggregation. The genetic and structural features of the PMEC932-7 cell line suggest the possibility of its application as an in vitro validation system to confirm the expression of mammary gland-specific transgenic vectors for the generation of transgenic pigs.

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<Research interests>

Animal reproductive endocrine mechanism, Artificial insemination, Pregnancy system