ELSEVIER

Cell Biology International 29 (2005) 576–582

Cell Biology International

www.elsevier.com/locate/cellbi

Gene transfection and expression in a primary culture of mammary epithelial cells isolated from lactating sows

Y.L. Sun [a], C.S. Lin [b], Y.C. Chou [a,*]

[a] *Division of Biotechnology, Animal Technology Institute Taiwan, P.O. Box 23, Chunan 350, Miaoli, Taiwan, ROC*
[b] *Department of Biological Science and Technology, National Chiao Tung University, 75 Po-Ai Street, Hsinchu 300, Taiwan, ROC*

Received 1 October 2004; revised 24 January 2005; accepted 2 March 2005

Abstract

Porcine mammary epithelial cells (PMECs) were isolated from lactating sow mammary glands and cultured on a matrix gel. Primary culture cells expressed significant amounts of the specific marker cytokeratin as determined by immunohistochemistry, and exhibited mammary-specific functions, such as transcription of α-lactalbumin, β-casein and β-lactoglobulin genes. They also formed mammospheres when the medium was supplemented with lactogenic hormones. The PMECs were used to study gene transfer and expression in vitro. A gene encoding enhanced green fluorescent protein (EGFP) was used as a reporter and two constructs were investigated, pEGFP-N1 (a vector constructed with a CMV promoter followed by the EGFP gene) and pGB562/GFP (a mammary gland-specific expression vector with regulatory sequences from the goat β-casein gene linked to EGFP). The efficiency of DNA transfer into the cultured PMECs was about 20–30%. GFP expression in the pGB562/GFP-transfected PMECs was markedly stimulated by prolactin supplements in the medium. The established PMECs maintained optimal gene expression from 1 to 20 passages and appeared to provide an efficient and convenient system for assessing the expression of transgenes containing mammary gland-specific promoters.

Keywords: Mammary epithelial cells; Pig; Gene transfection; Gene expression

1. Introduction

Milk has several advantages over prokaryotic and yeast systems for expressing transgenes encoding complex proteins (Zhang et al., 1998). Exogenous recombinant proteins secreted into milk have undergone appropriate post-translational modifications (e.g., carboxylation and glycosylation) in vivo, making them structurally and functionally identical to the native proteins (Houdebine, 1995, 2000). Mice have been used as a model but mouse milk has very limited availability. Cow and goat mammary glands provide good candidates for use as bioreactors for this purpose, because of high milk production (Janne et al., 1998; Niemann and Kues, 2003; Wheeler, 2003). Pigs are alternative candidates; their advantages include short gestation period, large litter size and small breeding area requirements (Wheeler and Walters, 2001). In addition to pharmaceutical applications, transgenic alteration of sow milk could be used to enhance piglet health and growth performance (Wheeler et al., 2001).

Abbreviations: PMECs, porcine mammary epithelial cells; EGFP, enhanced green fluorescent protein; rEGFP, recombinant enhanced green fluorescent protein; RT-PCR, reverse transcription-polymerase chain reaction; α-La, α-lactalbumin; β-ca, β-casein; β-Lg, β-lactoglobulin; BMECs, bovine mammary epithelial cells.

* Corresponding author. Tel.: +886 37 585826; fax: +886 37 585829.
E-mail address: chouyc@mail.atit.org.tw (Y.C. Chou).

1065-6995/$ - see front matter © 2005 International Federation for Cell Biology. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.cellbi.2005.03.021

The main barrier to obtaining transgenic livestock is modest transgene expression. An in vitro or ex vivo screening system that identifies superior transgenes prior to transfer could improve transgenic animal production. To increase transgene product recovery from transgenic pig milk, it would be desirable to use fully functional and transfectable porcine mammary epithelial cells for rapid screening. However, studies on porcine mammary gland to date have been limited to those using culture explants (Jerry et al., 1989; Simpson et al., 1998). Kumura et al. (2001) established a primary culture of porcine mammary epithelial cells (PMECs) as a model system for evaluating milk protein expression. Establishing mammary epithelial cell cultures is critical for studies of the mechanism of lactogenic hormone action and can also provide a simple, biologically meaningful in vitro test system for recombinant DNA constructs expressed in the mammary glands of transgenic animals.

In the present investigation, we isolated and cultured PMECs and used them to evaluate the efficiency of gene transfection and expression of tissue-specific gene constructs.

2. Materials and methods

2.1. Cell preparation and culture

Porcine mammary epithelial cells (PMECs) were isolated from the mammary gland of a lactating Landrace pig (Animal Technology Institute Taiwan, ATIT) by a simplified version of the method of Kumura et al. (2001) and all experiments were performed in accordance with the NIH guidelines of the Care and Use of Laboratory Animals (NIH Guidelines, 1996). Briefly, a 1 cm^3 sample of mammary tissue was minced using surgical scissors and the tissue pieces were dissociated by gentle agitation at 37 °C for 1.5–2 h in Dulbecco's phosphate buffered saline (D-PBS; GIBCO, Rockville, MD, USA) containing 0.1% collagenase A (Type III; Sigma, Louis, MO, USA), 0.05% hyaluronidase (Sigma), 2.4 U/ml dispase II (GIBCO) and 1× PSN antibiotics (50 μg/ml penicillin G, 50 μg/ml streptomycin sulfate, 10 μg/ml neomycin sulfate; GIBCO). After filtration through stainless steel meshes (80- and 100-mesh), to remove undissociated tissue and debris, the cells were collected by centrifugation at $800 \times g$ for 20 min and washed three times with D-PBS.

The isolated PMECs were cultured and maintained as previously reported (Kumura et al., 2001; Wu et al., 2003). The PMECs were cultured on a collagen matrix (5 μg/cm^3, BD Biosciences Clontech, Palo Alto, CA, USA) in basal medium, which comprised DMEM/F12 (GIBCO) containing 10% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, IL), insulin (10 μg/ml; Sigma), hydrocortisone (1 μg/ml; Sigma) and 1× PSN antibiotics. Approximately 5×10^6 cells were seeded and cultured on 9 cm plastic dishes coated with the collagen matrix at 37 °C in a humidified atmosphere containing 5% CO_2. Attached PMECs were washed several times with D-PBS containing 1× PSN antibiotics after 24 h, and fresh basal medium was added for continuous culturing. When subculturing, the PMECs were treated with 0.25% trypsin–EDTA (GIBCO) and incubated at 37 °C until the cells detached from the plastic dish. Then, the cells were centrifuged at 1000 rpm for 10 min. After centrifugation, the supernatant was removed and the cell pellet resuspended in basal medium for culturing.

2.2. Prolactin responsiveness

For hormonal induction of milk protein gene expression, 80% confluent cells were cultured on BD Matrigel™ gel (BD Biosciences Clontech) in basal medium containing prolactin (5 μg/ml; Sigma) and analyzed by reverse transcription-polymerase chain reaction (RT-PCR) using total PMECs RNA. The PCR primers for amplification of α-lactalbumin, β-casein, and β-lactoglobulin were as follows:

(1) α-Lactalbumin (GenBank accession no.: M80520)
α-La-F (22-mers) 5′-ATG GCT ATG GAG ACA TCA CTT T-3′
α-La-R (21-mers) 5′-ACA TCT TCT CAC AGA GCC ACT-3′
(2) β-Casein (GenBank accession no.: X54974)
β-ca-F (21-mers) 5′-CCA AAG CTA AGG AGA CCA TTG-3′
β-ca-R (19-mers) 5′-CAA CTG GTT GAG GCA CAG G-3′
(3) β-Lactoglobulin (GenBank accession no.: X54976)
β-Lg-F (19-mers) 5′-CCT GAA GGC GTA TGT GGA G-3′
β-Lg-R (17-mers) 5′-CAT GGG CAC AGA CAG GC-3′

Total cellular RNA preparation and RT-PCR were performed as previously described (Lin et al., 2002). Total RNA was extracted from PMECs (1×10^6 cells) using Trizol (GIBCO) in accordance with the manufacturer's instructions. Five micrograms of total RNA was reverse transcribed using 2.5 μM oligo-dT primers (Promega, USA), 1 mM of each dNTP (Promega), 20 U ribonuclease inhibitor (HT Biotechnology) and 5 U reverse transcriptase (RTase) (HT Biotechnology) in RT buffer (25 mM Tris–HCl, pH 8.3, 50 mM KCl, 2 mM DTT, 5 mM $MgCl_2$) in a total volume of 20 μl at 39 °C for 60 min. For each PCR reaction, 3 μl of RT product was added to a final volume of 50 μl containing 10 mM Tris–HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% Triton X-100, 0.001% gelatin, 200 μM of

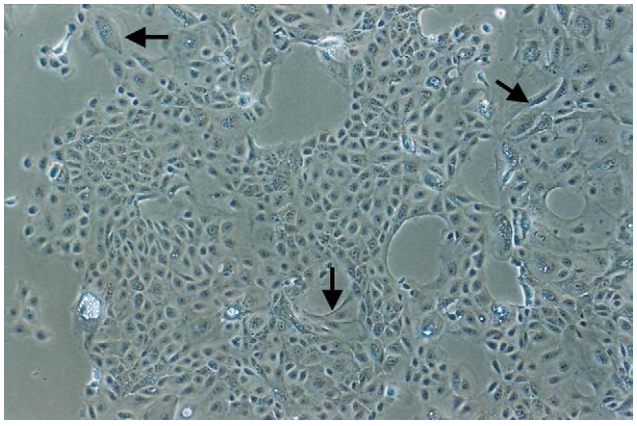

Fig. 1. Inverted photomicrographs of cultured porcine primary mammary epithelial cells (PMECs). PMECs were isolated from lactating mammary glands by enzymatic dissociation and subcultured continuously. PMECs cultured for 10 days (second passage) were the same as epithelial cultures (cobblestone morphology), with few contaminating fibroblasts (indicated by arrows). Magnification, 100×.

each dNTP, 0.5 U *Taq* DNA polymerase (HT Biotechnology) and 0.2 μM of each primer pair. For assay of gene expression, PCR was carried out in an Applied Biosystem DNA thermal cycler for 30 cycles of 30 s denaturation at 94 °C, 45 s annealing at 55 °C and 45 s extension at 72 °C.

2.3. Immunohistochemistry

Monoclonal antibodies against cytokeratin AE3 (Type II, subfamily no. 1–8; Chemicon, Temecula, CA, USA) (Huynh et al., 1991) and fibroblast antigen (Oncogene Research Products, San Diego, CA, USA) were used to characterize the epithelial cells. Cells were seeded in 6-well plates (1×10^5 well) for 24 h and then fixed in 95% ethanol for 1 min. Twenty microliters of mouse anti-bovine cytokeratin AE3 antibody and mouse anti-human fibroblast antigen, diluted 1:10 and 1:20, respectively, were applied per well and the plates were incubated at 37 °C for 1 h in a 100% humidity chamber. The plates were then washed (3 × 5 min) with D-PBS at room temperature and the cells were incubated with secondary antibody using a Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacturer's recommendations. The incubation and washes were as described above. Finally, the cells were stained with Vector

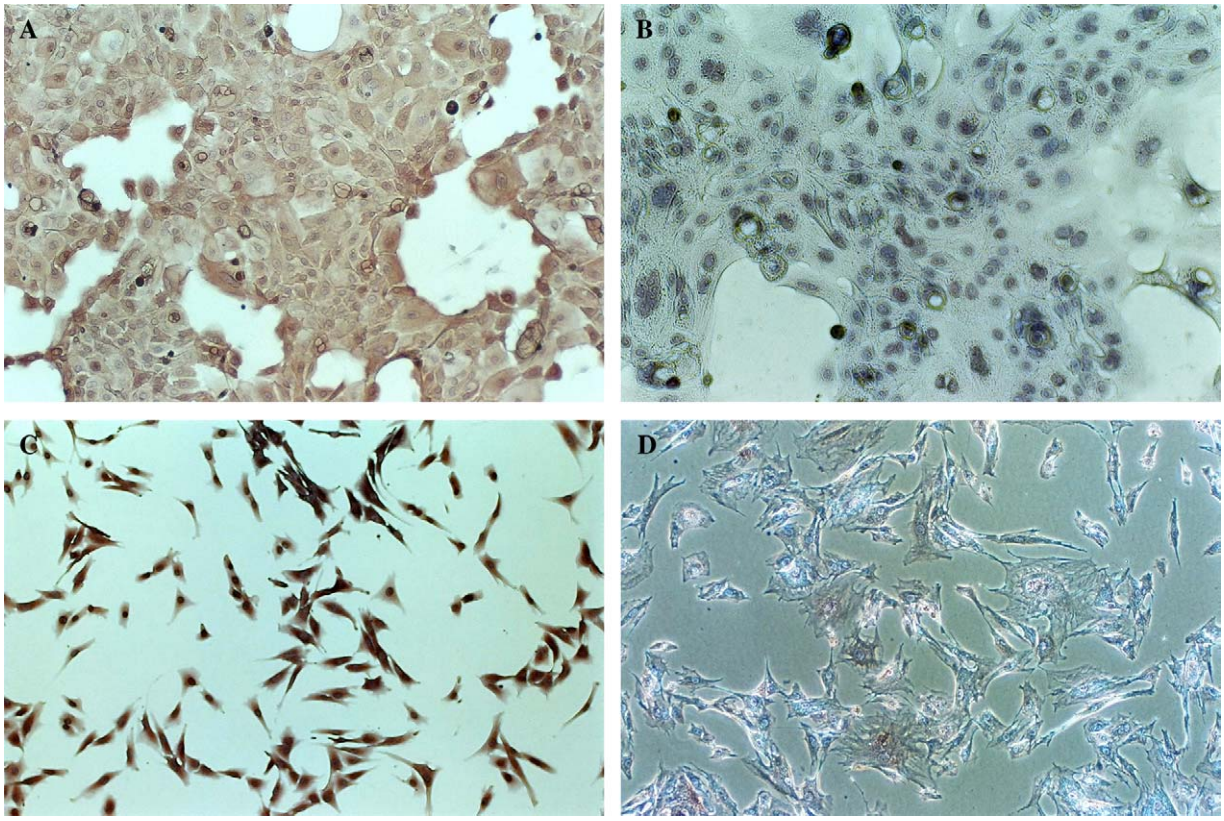

Fig. 2. Immunohistochemical identification of PMECs. The cultured PMECs at passage 6 were stained with a diluted (1:10) anti-cytokeratin antibody, cytokeratin AE3, specific for epithelial cells (A); no staining was visible when a diluted (1:20) anti-fibroblast antigen was used (B). In contrast to the PMECs, the fibroblasts (passage 6) isolated from porcine mammary gland were immunopositive to the anti-fibroblast antigen antibody (C) but immunonegative to the anti-cytokeratin AE3 antibody (D).

NovaRED Substrate Kit (Vector Laboratories, Inc.) and examined by light microscopy.

2.4. Gene transfection

Cells were transfected with plasmid DNAs using a Transfection Reagent Selector Kit according to the manufacturer's recommendations (Qiagen, Hilden, Germany): pEGFP-N1 [a vector constructed with a gene fragment encoding enhanced green fluorescent protein (EGFP) driven by a CMV promoter (BD Biosciences Clontech)]; and pGB562/GFP. pGB562/GFP, a mammary gland-specific expression vector, has the 5′ flanking sequence and intron 1 of the goat β-casein gene linked to the EGFP gene as a reporter (Wu et al., 2003). The cells were seeded at a density of $1-2 \times 10^5$/60 mm culture dish and grown overnight in basal medium, then washed with D-PBS to remove the remaining medium. At this stage, the culture medium was replaced with 1.5 ml of basal medium containing 0.2 μg plasmid DNA–liposome mixture and incubated at 37 °C for 4 h. The medium was then replaced with fresh basal medium and the cells were cultured for 2 days at 37 °C in a 5% CO_2 atmosphere. The transfected PMECs were observed using a fluorescence microscope (Axiovert 135; Carl Zeiss, Gottingen, Germany) with fluorescein isothiocyanate (FITC) optics.

2.5. Quantitative fluorometric assay

GFP fluorescence was assayed as described previously (Wu et al., 2003). The cells were washed twice with PBS and then carefully removed from the Matrigel-coated dish with 0.05% trypsin–EDTA. The cell pellets were resuspended in lysis buffer (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl, pH 8.0) and freeze/thawed five times in a dry ice/ethanol bath. The lysate was centrifuged at 12,000 × *g* for 5 min at 4 °C and the supernatant was assayed for GFP expression using a TD 700 Fluorometer (Turner Designs, Sunnyvale, CA, USA) as described in the manual. A standard curve was drawn using purified recombinant enhanced green fluorescent protein (rEGFP; BD Biosciences Clontech).

2.6. Statistical analysis

All quantitative data were expressed as mean ± standard deviation (SD) and the difference between the means of two groups was assessed by ANOVA (Hocquette and Brandstetter, 2002). *P* values below 0.05 were considered statistically significant.

3. Results and discussion

A high yield of PMECs was obtained from fresh mammary glands by the collagenase dissociation method. The cells took about 4–5 days to begin adhering, spreading and growing on the collagen matrix. They grew well in the basal medium and exhibited the characteristic cobblestone morphology of epithelial cells for about two passages (Fig. 1). The initial preparation was contaminated with fibroblast-like cells but these were absent after five passages. The contaminating fibroblast cells totaled less than 5% of the mammary epithelial cells after four passages. Two passages after starting the culture, most of the cell populations comprised morphologically uniform epithelial-like cells with 18- to 24-h doubling times. After 15–20 passages, the cells became larger, grew more slowly and exhibited a more vacuolated morphology. Cultures were not used for further experiments after these indications of senescence appeared.

To characterize the isolated PMECs, an 80% confluent monolayer was stained with cell-type-specific primary antibodies followed by secondary antibodies. The primary PMECs culture stained positively with an anti-cytokeratin antibody specific for epithelial cells (Fig. 2). This result demonstrated that the PMECs expressed the epithelial cell marker cytokeratin AE3. Absence of staining with an anti-fibroblast antigen

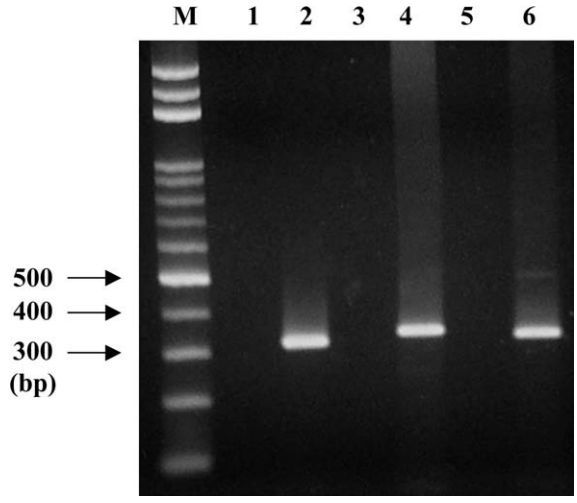

Fig. 3. Hormone responsiveness of the expression of porcine milk protein genes. Total RNA isolated from PMECs at passage 6 cultured on Matrigel in basal medium with or without 5 μg/ml prolactin was used to investigate the expression of porcine milk protein genes, amplified by the RT-PCR method. M, a 100-bp ladder as a marker; lanes 1 and 2, α-lactalbumin; lanes 3 and 4, β-casein; lanes 5 and 6, β-lactoglobulin. The PMECs were cultured in basal medium without prolactin (5 μg/ml) (lanes 1, 3, and 5) and with prolactin (lanes 2, 4, and 6). The sizes of the amplified fragments for α-lactalbumin, β-casein and β-lactoglobulin were predicted to be 320, 341, and 328 bp, respectively.

marker showed that these primary cells were not fibroblasts.

Immortalized cell lines derived from mammalian mammary gland via viral element transfection or carcinogen treatment, such as NMuMG, COMMA-D, HC-11 and MAC-T cells, have been successfully cultivated on matrix gels. These cells have generally been used for in vitro studies to test the expression capacity of milk gene promoters or transgenes (Altiok and Groner, 1993; Doppler et al., 1995; Winklehner-Jennewein et al., 1998; Wu et al., 2003). However, these cell lines express very different characteristics from their natural counterparts. For example, lipopolysaccharide inhibits the proliferation of primary bovine mammary epithelial cells (BMECs) but not of MAC-T cells, which are BMECs transfected with SV-40 large T antigen (Calvinho et al., 2001). Endogenous milk proteins were induced rapidly by lactogenic hormones in HC-11 cells, with no requirement for extracellular matrix components (Doppler et al., 1989; Groner and Gouilleux, 1995). However, cultured HC-11 cells lack the three-dimensional alveolar-like structure of lactating mammary glands. Despite the requirement of NMuMG cells for Matrigel, these cells have been used to investigate molecular mechanisms (Vihinen et al., 1996; Nemir et al., 2000; Wechselberger et al., 2001). Wu et al. (2003) showed that, under appropriate culture conditions, NMuMG simulated the three-dimensional in vivo morphology.

To indicate the status of PMEC differentiation, the cells were cultured on a Matrigel-coated dish and basal medium containing prolactin was supplied. The cells aggregated to form blisters or secretory dome structures on the Matrigel basement membrane (data not shown). To evaluate the extent of differentiation of the PMECs in vitro, expression of the α-lactalbumin, β-casein and β-lactoglobulin genes was measured with or without prolactin stimulation. With the hormone supplement, α-lactalbumin, β-casein and β-lactoglobulin mRNAs were detected; without the supplement, none of the three was detectable (Fig. 3).

pEGFP-N1 and pGB562/GFP-transfected PMECs were cultured in basal medium with or without prolactin to test the efficiency of gene transfection and expression. About 20–30% of the undifferentiated PMECs (without prolactin supplement) expressed GFP. However, no GFP expression was detected in cells transfected with pGB562/GFP (data not shown). Prolactin-treated

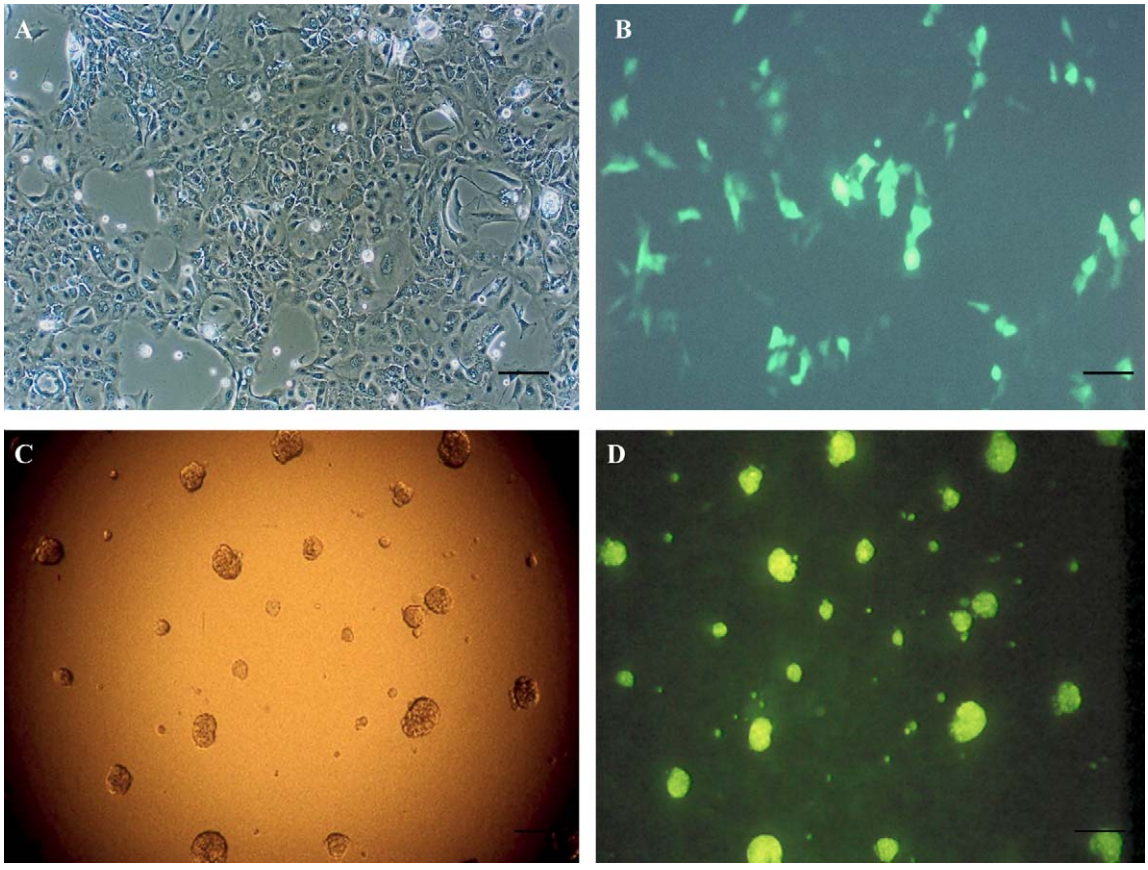

Fig. 4. Gene transfection and GFP expression in PMECs. The six passages of PMECs transfected with pEGFP-N1 by liposome were cultured in basal medium for 24 h. (A) Image under bright field; (B) image under fluorescent field. The six passages of PMECs transfected with pGB562/GFP were cultured in basal medium with prolactin supplement for 24 h. (C) Image under bright field; (D) image under fluorescent field. Bar indicates 200 μm. Magnification, 200×.

PMECs transfected with pEGFP-N1 or pGB562/GFP showed high GFP expression (Fig. 4). The levels of GFP in PMECs transfected with pGB562/GFP cultivated with prolactin at 1, 5, 10, 15 and 20 passages were 5.5 ± 1.2, 11.3 ± 2.0, 12.1 ± 1.58, 9.4 ± 1.9, and 5.1 ± 1.0 μg/mg, respectively (Fig. 5).

We isolated a primary culture of porcine mammary epithelial cells (PMECs) with little fibroblast contamination by enzymatic dissociation and continuous subpassaging. PMECs cultivated on Matrigel formed a hollow alveolar-like structure with numerous apical microvilli. Matrigel is an extract from Engelbreth–Holm–Swarm tumor and contains laminin, collagen IV and entactin. "Lumen" formation, apical microvilli and well-developed rough endoplasmic reticulum provide evidence that mammary cells cultivated on extracellular matrix components are capable of secretory activity after hormone induction (Li et al., 1987).

Hormone interactions are responsible for inducing milk protein gene transcription. Doppler et al. (1990) indicated that dexamethasone increases the sensitivity of mammary gland cells to prolactin; the sensitivity increase is slow and can be rapidly reversed by withdrawal of dexamethasone. In this study, we found that prolactin was strongly synergistic, increasing gene expression up to 10-fold compared with transfected PMECs maintained in the presence of insulin and hydrocortisone. This indicated that the established PMEC cultures might be suitable for studying milk protein gene expression.

In conclusion, the primary PMEC cultures established in this investigation maintained biological functions such as transcription of mammary-specific genes, including α-lactalbumin, β-casein and β-lactoglobulin, when supplemented with lactogenic hormone. The cells appeared efficient and convenient for evaluating the function of a tissue-specific promoter and determining whether the constructs are suitable for generating transgenic pigs.

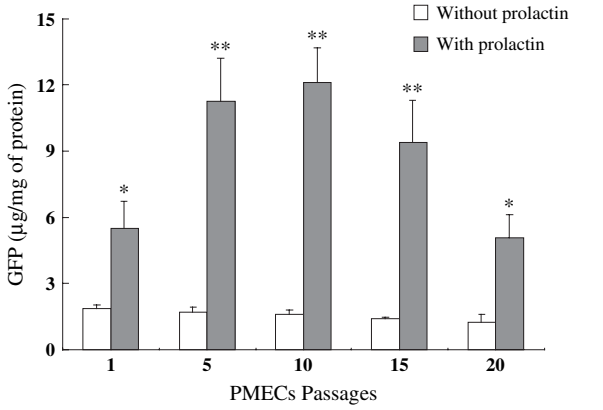

Fig. 5. Effect of passage on GFP expression by pGB562/GFP-transfected PMECs. The cells were cultured in basal medium with or without 5 μg/ml prolactin for 24 h before GFP determination. The value of each passage was determined by a fluorometric assay on three independent cultures, with each bar indicating mean ± SD. * and ** indicate $P < 0.01$ and $P < 0.001$ compared to the pGB562/GFP-transfected PMECs of the same passage without prolactin supplement, respectively.

Acknowledgements

The authors gratefully acknowledge Prof. M.C. Huang (National Chung Hsing University, Taiwan) for the gift of the pGB562/GFP plasmid, and Dr. H.T. Wu (National Laboratory Animal Center of National Applied Research Laboratories, Taiwan) for providing help with the fluorescence technology. This work was supported by grant 92-EC-17-A-17-R7-0454 from the Ministry of Economic Affairs of Executive Yuan, Taiwan, ROC.

References

Altiok S, Groner B. Interaction of two sequence-specific single-stranded DNA-binding proteins with an essential region of the beta-casein gene promoter is regulated by lactogenic hormones. Mol Cell Biol 1993;13:7303–10.

Calvinho LF, Almeida RA, Oliver SP. Influence of bacterial factors on proliferation of bovine mammary epithelial cells. Rev Argent Microbiol 2001;33:28–35.

Doppler W, Groner B, Ball RK. Prolactin and glucocorticoid hormones synergistically induce expression of transfected rat beta-casein gene promoter constructs in a mammary epithelial cell line. Proc Natl Acad Sci U S A 1989;86:104–8.

Doppler W, Hock W, Hofer P, Groner B, Ball RK. Prolactin and glucocorticoid hormones control transcription of the beta-casein gene by kinetically distinct mechanisms. Mol Endocrinol 1990;4:912–9.

Doppler W, Welte T, Philipp S. CCAAT/enhancer-binding protein isoforms beta and delta are expressed in mammary epithelial cells and bind to multiple sites in the beta-casein gene promoter. J Biol Chem 1995;270:17962–9.

Groner B, Gouilleux F. Prolactin-mediated gene activation in mammary epithelial cells. Curr Opin Genet Dev 1995;5:587–94.

Hocquette JF, Brandstetter AM. Common practice in molecular biology may introduce statistical bias and misleading biological interpretation. J Nutr Biochem 2002;13:370–7.

Houdebine LM. The production of pharmaceutical proteins from the milk of transgenic animals. Reprod Nutr Dev 1995;35:609–17.

Houdebine LM. Transgenic animal bioreactors. Transgenic Res 2000;9:305–20.

Huynh HT, Robitaille G, Turner JD. Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. Exp Cell Res 1991;197:191–9.

Janne J, Alhonen L, Hyttinen JM, Peura T, Tolvanen M, Korhonen VP. Transgenic bioreactors. Biotechnol Annu Rev 1998;4:55–74.

Jerry DJ, Stover RK, Kensinger RS. Quantitation of prolactin-dependent responses in porcine mammary explants. J Anim Sci 1989;67:1013–9.

Kumura H, Tanaka A, Abo Y, Yui S, Shimazaki K, Kobayashi E, et al. Primary culture of porcine mammary epithelial cells as a model system for evaluation of milk protein expression. Biosci Biotechnol Biochem 2001;65:2098–101.

Li ML, Aggeler J, Farson DA, Hatier C, Hassell J. Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. Proc Natl Acad Sci U S A 1987;84:136–40.

Lin CS, Wu YC, Sun YL, Huang MC. Postnatal expression of growth/differentiation factor-8 (GDF-8) gene in European and Asian pigs. Asian-Australas J Anim Sci 2002;9:1244–9.

Nemir M, Bhattacharyya D, Li X, Singh K, Mukherjee AB, Mukherjee BB. Targeted inhibition of osteopontin expression in the mammary gland causes abnormal morphogenesis and lactation deficiency. J Biol Chem 2000;275:969–76.

Niemann H, Kues WA. Application of transgenesis in livestock for agriculture and biomedicine. Anim Reprod Sci 2003;79:291–317.

NIH Guidelines. Guide for the care and use of laboratory animals. Washington, DC: Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, National Academy Press; 1996.

Simpson KJ, Bird P, Shaw D, Nicholas K. Molecular characterization and hormone-dependent expression of the porcine whey acidic protein gene. J Mol Endocrinol 1998;20:27–35.

Vihinen T, Maatta A, Jaakkola P, Auvinen P, Jalkanen M. Functional characterization of mouse syndecan-1 promoter. J Biol Chem 1996;271:12532–41.

Wechselberger C, Ebert AD, Bianco C, Khan NI, Sun Y, Wallace-Jones B, et al. Cripto-1 enhancers migration and branching morphogenesis of mouse mammary epithelial cells. Exp Cell Res 2001;266:95–105.

Wheeler MB, Walters EM. Transgenic technology and applications in swine. Theriogenology 2001;56:1345–69.

Wheeler MB, Bleck GT, Donovan SM. Transgenic alteration of sow milk to improve piglet growth and health. Reprod Suppl 2001;58:313–24.

Wheeler MB. Production of transgenic livestock: promise fulfilled. J Anim Sci 2003;81(Suppl. 3):32–7.

Winklehner-Jennewein P, Geymayer S, Lechner J, Welte T, Hansson L, Geley S, et al. A distal enhancer region in the human beta-casein gene mediates the response to prolactin and glucocorticoid hormones. Gene 1998;217:127–39.

Wu HT, Lin CS, Huang MC. In vitro and ex vivo green fluorescent protein expression in alveolar mammary epithelial cells and mammary glands driven by the distal 5′-regulative sequence and intron 1 of the goat beta-casein gene. Reprod Fertil Dev 2003;15:231–9.

Zhang K, Lu D, Xue J, Huang Y, Huang S. Construction of mammary gland-specific expression vectors for human clotting factor IX and its secretory expression in goat milk. Chin J Biotechnol 1998;13:271–6.