ISOLATION AND CHARACTERIZATION OF PORCINE MAMMARY EPITHELIAL CELLS FROM NON-LACTATING AND NON-PREGNANT GILT

A Thesis

by

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ABSTRACT

Mammary epithelial cells (MEC) are the functional units of the mammary gland during lactation. There are well-established MEC cell lines, such as bovine MAC-T, as well as mouse and human cell lines. Primary MEC lines are useful models to understand metabolism, growth, differentiation and other functions of the mammary gland epithelium. It was recently reported that dietary supplementation with either branched-chain amino acids or glutamate enhanced milk production by sows. The availability of a stable porcine MEC (PMEC) cell line is important to elucidate the underlying mechanisms responsible for effects of amino acids on enhancing milk production by lactating sows. In the present study, PMEC were isolated from mammary glands of a 9-month-old non-pregnant and non-lactating female pig. Cells were then cultured at $37^{o}C$ and 5% CO_2 in 10 ml Dulbecco's Eagle Medium-F12 medium containing 5 µg/ml insulin, 1 µg/ml hydrocortisone, 5 ng/ml epidermal growth factor, 50 µg/ml Gentamycin, 5% fetal calf serum, and antifungal and antibiotics from Gibco.

The PMEC plated on culture plates displayed a monolayer with a cobblestone epithelial-like morphology and formed island monolayer aggregates with typical characteristics of MEC. PMEC cells expressed cytokeratin-18, but not Vimentin. These cells also expressed the mammary gland-specific gene for beta-casein and synthesized and released beta-casein protein into the culture medium, based on results of reverse transcription polymerase chain reaction and western blot analyses.

DEDICATION

I dedicate this thesis to my wonderful family, especially, to my father and mother for instilling the importance of hard work and higher education.

ACKNOWLEDGEMENTS

NOMENCLATURE

DMEM   Dulbecco's Eagle Medium

DMSO   Dimethyl sulfoxide

EDTA   Ethylene Diamine Tetra-ascetic Acid

EGF   Epidermal Growth Factor

FCS   Fetal Calf Serum

GAPDH   Glyceraldehyde-3-Phosphate Dehydrogenase

HEPES   4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC   High Performance Liquid Chromatography

MEC   Mammary Epithelial Cells

MTT   3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PBS   Phosphate Buffered Saline

PMEC   Porcine Mammary Epithelial Cells

PSN   Penicillin, Streptomycin, and Neomycin

RTPCR   Reverse Transcriptase-Polymerase Chain Reaction

TBS   Tris-Buffered Saline

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CHAPTER I

INTRODUCTION TO PORCINE MAMMARY EPITHELIAL CELLS

Mammary epithelial cells (MEC), also called alveolar cells, synthesize and secrete milk proteins, lipids, and lactose (Manjarin et al., 2012). These cells express cytokeratin-18 (an intermediate filament specific for epithelial cells) while uniquely producing β-casein (Bartek et al., 1985; Parry et al., 1987). In response to prolactin, MEC undergo coordinated changes in proliferation and differentiation during gestation and lactation (Malewski et al., 2002). Therefore, prolactin plays an important role in initiating and maintaining lactogenesis in mammals.

A primary MEC cell line is a useful model system to understand how metabolism, growth and differentiation of mammary cells are regulated by amino acids as well as an expression/selection system for producing protein pharmaceuticals (Link et al., 2006). Milk protein genes are transcribed in the mammary gland of lactating animals, and the encoded proteins are secreted in large quantities into milk (Malewski et al., 2002). Synthesis of milk proteins is induced by prolactin, glucocorticoids and insulin in MEC (Manjarín et al., 2012).

Beta-lactoglobulin and whey acid protein (WAP) are expressed as major whey protein components in porcine MEC (Simpson et al., 1998). However, WAP proteins that are abundant milk proteins in mice (Piletz et al., 1981), but they have not been reported in milk from swine or humans (Hennighausen et al., 1982). The polypeptide hormone prolactin is synthesized by lactotroplhs in the anterior pituitary gland under multi-hormonal control and it is a member of the growth hormone family. Prolactin

regulates ductal and alveolar growth of the mammary epithelium, and controls differentiation of the mammary gland by initiating and maintaining lactogenesis (Topper and Freeman et al., 1980). Moreover, prolactin exerts its biological effects via a plasma membrane receptor (Kelly et al., 1984; Djiane et al., 1985; Haldosen and Gustafsson et al., 1987).

Cell phenotypes can be characterized effectively by immunohistochemistry. Positive staining with antibodies directed to cytokeratins, a epithelial cell marker, identify the cells in culture as epithelial lineage (Gebert et al., 1994).

Branched chained amino acid metabolism (Wu et al., 2013) plays an important role in mammary epithelial cell physiology during lactation. Lactating sows have high requirements for branched-chain amino acids (BCAA), leucine, isoleucine, and valine to support milk production (Kim et al., 2009) and the mammary gland contains high concentrations of free and peptide bound glutamine plus glutamate (Wu et al., 1994 and Davis et al., 1994).

During lactation, MEC synthesize glutamate, glutamine and aspartate from branched-chain amino acids (BCAA), leucine (Leu), isoleucine (Ile) and valine (Val). BCAA are actively taken up and catabolized (Brosnan et al., 2012; Lei et al., 2012) by the mammary gland. It has been reported that the concentration of free Glutamate (Glu) in rat's milk is approximately 10 times higher than that in plasma.

Previous work has also shown that mammary tissue actively transports Leu from blood, one of the three BCCA and metabolize Leu via transamination to form other amino acids such as Glu and aspartate (Asp). The uptake of BCAA from plasma by

lactating porcine mammary gland substantially exceeds their output in milk, whereas Gln output is 125% greater than its uptake from plasma (Kim SW et al., 2009). Thus, BCAA play an important role in the synthesis of Glu, Gln, Asp, alanine (Ala), and asparagine (Asn) by MEC, which are secreted into milk (Li, et al., 2009). This explains the abundance of these amino acids in the milk of mammals (Wu and Knabe 1994; Lei et al. 2012).

MAMMARY EPITHELIAL CELL GROWTH AND DEVELOPMENT

In mammals, the synthesis and secretion of milk for the nourishment of newborn offspring are directly associated with a female organ called the mammary gland (Neville et al., 2012).   Proteins, lipids, sugars, and water are major constituents enriched in milk to provide the primary source of nutrients for newborns before they are able to digest more solid foods. The provision of milk is significant for the optimal growth and development of the neonatal small intestine (Lei et al., 2012).

The presence of mammary glands and the production of milk by female mammals provides a period of intense maternal interaction with the newborn during early behavioral development.   This bonding is required for optimal neurological development and health in neonates.

The mammary gland or mammary duct is made up of two components: the parenchyma and the surrounding stroma. The parenchyma is composed of epithelial structures, alveoli, and ducts to synthesize and secret milk (Lei et al., 2012). The stroma provides a framework for supporting and regulating function of the epithelial cells. The parenchyma of the alveoli appears like a cluster of grapes in which

milk is stored, and the branching ducts are tubular canals carrying glandular secretions. Each duct serves a specific lobule and forms a tree-like branched system, which connects to nipples. The branching ductal system is comprised of two types of cells; myoepithelial cells and a single layer of inner epithelial cells, both of which are essential to production of milk.

MORPHOLOGY OF PORCINE MAMMARY EPITHELIAL CELLS (PMEC)

Previous morphological studies on PMEC revealed that when they were grown at low density on a plastic substratum, the cells had a characteristic cobblestone morphology and formed individual island-like structures, and each of the islands merged with the nearest island as they grew to confluency (Zhao et al., 2010) There were also reports that subcultured immortalized PMEC (SI-PME) proliferated without changes in morphology or growth pattern, with an estimated population doubling time of 24 hours and with increasing density as they organized into lumen-like structures with elongated cells at the margins (Chou et al.2001). SI-PMEC cells from stocks frozen at Passage 30 were subcultured up to 20 times without changes in viability, proliferation rate, or morphology.

PROLACTIN

The major function of MEC is to secrete milk during the lactation period. These cells undergo coordinate changes in growth and differentiation throughout pregnancy, lactation and involution following lactation (Desprez et al., 1998). PMEC lines are useful model systems to understand how metabolism, growth and differentiation of mammary cells are regulated by amino acids, as well as an providing for an

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expression/selection system for producing important pharmaceutical proteins (Aoki, 2006). Milk protein genes are transcribed in the mammary gland of lactating animals, and encode for proteins that are secreted in large quantities into milk (Malewski et al., 2002).

The polypeptide hormone prolactin is synthesized by lactotroph cells in the anterior pituitary gland under multi-hormonal control and it is a member of the growth hormone family (Freeman et al., 2000). Prolactin regulates ductal and alveolar growth of the mammary epithelium and controls differentiation of the mammary gland by initiating and maintaining lactogenesis (Rosen, 2012). Moreover, prolactin exerts its biological effects via a plasma membrane receptor.

Growth hormone is a polypeptide hormone is synthesized in pituitary gland which is a member of growth hormone family (Hizuka et al., 1982). It is synthesized in different tissues of the body such as the pituitary gland, within the central nervous system, in immune system, in the uterus and its associated tissues of conception, and even the mammary gland itself. Until recently, the biological activity of prolactin was thought to be limited to reproduction (Freeman et al., 2000). However, numerous functions have been described for prolactin not only as a growth stimulatory signal but also as other stimulatory roles, such as the nursing stimulus (Gallego et al., 2001).

CYTOKERATIN 18

The organization of cytokeratins or intermediate filaments provides mechanical strength to epithelial cells as they form the intracytoplasmic cytoskeleton of epithelial cells. The term "cytokeratin" began to be used in the late 1970s. Cytokeratins are in the

intermediate filament (IF) protein family and cytokeratin 18 belongs to the type I cytokeratin family. Cytokeratins are intermediate filament proteins found in epithelial cells, including the mammary epithelium. Keratin gene expression is usually regulated by factors affecting differentiation of the epidermal cells within the stratifying squamous epithelium.

VIMENTIN

Vimentin is a a type III intermediate filament (IF) protein found in animals and encoded by the VIM gene. Vimentin protein is expressed in mesenchymal cells (Sudo et al., 2013). A central helical domain structure of the vimentin monomer is bounded on each end by a non-helical amino group and a carboxyl group. During the differentiation of cells, vimentin (57 kDa) is the most ubiquitous of the intermediate filament proteins and the first to be expressed (Mohammad et al., 2009). During differentiation, all primary cell types express vimentin except for most non-mesenchymal cells. Among the a wide variety of mesenchymal cell types, fibroblasts and endothelial cells express vimentin (Sonoshita et al., 2002).

ROLE OF BRANCHED-CHAIN AMINO ACIDS IN GROWTH AND DEVELOPMENT

BCAA metabolism plays an important role in mammary epithelial cell physiology during lactation (Rezaei et al., 2013). Lactating sows have high requirements for leucine, isoleucine, and valine to support milk production (Kim et al., 2009) and milk contains high concentrations of free and peptide bound glutamine plus glutamate (Wu et al., 1996). During lactation, BCAA are actively taken up and catabolized in MEC for syntheses of glutamate, glutamine, alanine and aspartate (Lei et al., 2013).

It has been reported that the concentration of free glutamate (Glu) in sow's milk is approximately 10 times higher than that in plasma (Wu and Knabe 1994). Mammary tissue actively takes up Leu from blood and Leu is one of the three BCCA that provide the α-amino group to synthesize other amino acids such as Glu, Asp, and Ala. These amino acids are then used by MEC for protein synthesis.

The uptake of BCAA from plasma by lactating porcine mammary glands substantially exceeds their output in milk, whereas Gln output is 125% greater than its uptake from plasma (Li et al., 2009). Leu, Ile, and Val are metabolized via transamination to produce Glu, Gln, Asp, Ala, and asparagine (Asn) to be secreted in milk. These amino acids had long been ignored in animal nutrition but they are now recognized as nutritionally essential for maximum growth, development and reproduction of animals (Hou et al. 2016).

COMPOSITION OF MILK PROTEIN

The total protein components of milk have been estimated by basic analytical techniques and the milk is now known to contain numerous specific proteins (Heck et al., 2009). The major group of milk proteins are the caseins which exist as a few subtypes in milk of most species. Milk protein composition is about 80% casein and 20% whey proteins (Dettori et al., 2015).

Quantitative analysis of cow's milk indicated that beta-lactoglobulin and alpha-lactalbumin are the major whey proteins (Anderson et al., 1982). In addition to the major milk proteins, (i.e., caseins) β-lactoglobulin and α-lactalbumin, are synthesized in the MEC. However, both serum immunoglobulin and albumin are synthesized by lymphocytes in the mammary tissue and these proteins cross the mammary epithelium and later become components in milk.

ROLE OF CASEIN PROTEIN IN MAMMALS

Phosphorylated casein proteins are commonly found in mammalian milk (Bingham, 1979) and, due to their specific amino acid composition, they play many important roles in growth and development of the newborn (Wu, 2010). Most human diets are supplemented with high quality milk proteins due to their ability to provide adequate nutrition for human growth. Similarly, sows milk provides casein to support the growth and development of neonatal pigs.

The small intestine is the major organ wherein caseins are digested to provide amino acids (Gardner, 1978). Whey proteins begin to be digested in the stomach and are relatively less digestible in the intestine. When substantial amounts of whey protein are

not digested fully in the intestine, some of the intact protein may stimulate a localized intestinal or a systemic immune response.   Micells are large molecular structures of milk proteins, such as casein, that form when dispersed in solvents. In milk, large amounts of calcium are bound to casein.

The micellular globular structure of milk casein is involved in digestion of milk in the stomach and intestine. It is also the basis for many products of the milk industries and the basis for the ability to easily separate some proteins and other components from cow's milk (Sodhi et al., 2012). Casein is one of the most abundant organic components of milk, in addition to lactose and milk fat.

Due to its clustering of polar residues and hydrophobic residues in different region of each molecule, casein alone is not very soluble in the aqueous environment of milk. However, casein is capable of forming micelle granules that are maintained as a colloidal suspension in milk. If the micellar structure is disturbed, the micelles may come apart and the casein may come out of solution, forming a gelatinous material. This is part of the basis for production of non-fluid milk products like cheese.

OTHER MILK PROTEINS

Milk protein genes are transcribed in the mammary gland of lactating animals, and the encoded proteins are secreted in large quantities into milk (Malewski et al., 2002). As noted previously, synthesis of milk proteins by MEC is induced by prolactin, glucocorticoid and insulin(Manjarín et al., 2012). Other milk proteins include immunoglobulins, enzymes (e.g., D-amino acid oxidase and L-amino acid oxidase),

hormones, growth factors (insulin-like growth factor), and regulatory proteins (e.g., osteopontin)

LIPID ANALYSIS BY OIL RED STAINING

Oil Red O, a nitrogen containing organic diazo dye with molecular formula of $C_{26}H_{24}N_4O$, is used widely in laborotary experiment for staining of natural triglycerides (TAG) and lipids (De Baets et al., 2010). It has the appearance of a red powder with light absorption at 518 nm. In this study, Oil red staining was used to veriify the presence of lipid droplets in MEC treated with prolactin. Oil red binds mainly fat deposits on the surface of porcine mammary epithelial cells.

The Oil Red O (ORO) stain can identify neutral lipids and fatty acids in secreted from cells and tissues. Fresh smears or cryostat sections of tissue or cells are necessary because fixatives containing alcohols, or routine tissue processing with clearing, will remove lipids. The ORO is a rapid and simple stain. The purpose of this study was to prospectively evaluate the release of milk due to prolactin induction of PME cells.

CHAPTER II

MATERIALS AND METHODS

REAGENTS

The RNA extraction reagent TRIzol, ThermoSCRIPT RT-PCR System, and CNBr Sepharose 4B were procured from Invitrogen Life Technologies (Carlsbad, CA). The IRDye 800-conjugated secondary antibody against rabbit IgG was purchased from LI-COR Bioscience (Lincoln, NE).

Dulbecco's modified Eagle Ham/F12 medium (DMEM/F12), antibiotic solution (penicillin-G, streptomycin, and neomycin [PSN, 100x]), phosphate-buffered saline (PBS), fetal bovine serum (FBS; charcoal striped), insulin solution (from bovine pancreas), triiodothyronine, transferrin, dexamethasone, and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). The 100x PSN contained 10,000 units penicillin-G, 10 mg streptomycin, and 20 mg neomycin per milliliter).

ISOLATION AND CULTURE OF PMEC

This experiment was approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University. Porcine mammary gland tissue was obtained from a 9-month-old non-pregnant and non-lactating gilt (F1 cross of Yorkshire X Landrace sow and Duroc X Hampshire boar) maintained at Texas A&M University's Swine Center (College Station, TX).

The non-lactating gilt was used because we found in our preliminary work that PMEC isolated from mammary glands of lactating sows did not survive after two

passages in culture. The gilt had free access to drinking water and was fed a corn- and soybean meal-based diet (2 x 1.5 kg/d), as described previously (Li et al., 2010).

On the day of collecting mammary glands, the gilt was placed in a restraining crate, administered an intramuscular dose of Telazol (2 mg/kg body weight; Zoetis Inc., Kalamazoo, MI) to induce anesthesia, and the field of two mammary glands in the middle of the mammary line of the abdomen was cleaned sequentially with warm water, Betadine (Purdue Products L.P., Stamford, CT), and 70% alcohol.

After euthanasia with intravenous administration of an over-dose pentobarbital (120 mg/kg BW), two mammary glands were obtained with scissors, placed in ice-cold sterile phosphate buffered saline (Sigma-Aldrich) containing 50 µg/ml gentamicin and 1X PSN, and immediately used for isolation of PMEC. All procedures for PMEC isolation were performed at 25°C in a sterile environment. Specifically, two mammary gland samples were disinfected with 75% ethanol and then washed thoroughly with PBS containing 50 µg/ml gentamicin and 1X PSN.

The tissues were cut into 1-2 cm^3 pieces, and all of the tissue pieces were pooled into one sterile 50-ml falcon centrifuge tube (Fisher Scientific, Houston, TX), and minced into 5 mm cubes using surgical scissors.

Thereafter, 40 ml DMEM/F12 digestion medium with 5% FBS, 1 mg/ml collagenase A (Cat # C9407; Sigma-Aldrich), 0.05% hyaluronidase (Cat # H3506; Sigma-Aldrich), 50 µg/ml gentamicin and 1X PSN (Sigma-Aldrich, city and state?) was added to the tube. The tissue pieces were dissociated by gentle agitation at 37°C for 12 h. After passing the solution of dissociated cells through a 70-100 µm filter (Cat #

352360; BD Biosciences, San Jose, CA) into a 50-ml conical tube and washing the filter with 10 ml buffer, the combined effluent (containing mainly PMEC and a small amount of fibroblasts) was collected.

The solution was centrifuged at $800 \times$ g for 3 min at 25°C. After the supernatant fluid was removed, the cell pellet was washed three times with PBS containing 1X PSN. At the above filtration step, the materials remaining on the filter that contained debris, undigested tissue, and organoids were discarded.

PMEC CULTURE, CELL PASSAGE, AND CRYOPRESERVATION

The isolated PMEC cells were resuspended and cultured in DMEM/F12 containing 10% FBS, 5 µg/ml insulin, 1 µg/ml hydrocortisone, 5 ng/ml epidermal growth factor (EGF), 50 µg/ml gentamicin, and 1X PSN. Approximately 1×10^6 cells were seeded and cultured in 6-well cell culture plates at 37°C in a humidified atmosphere containing 5% CO_2.

The culture plates were not coated with any protein or treated in any manner. The medium was changed every 24 h. When cells reached 90% confluence, they were washed with 1X PBS after the culture medium was removed. The cells were then trypsinized with 1X trypsin (a 0.25% trypsin solution with 1 mM EDTA), and split 1:4 or 1:5 for passaging.

The protocol used for the isolation and culture of PMEC was based on that for obtaining MEC from rodents (Qu et al., 2010).

We used gentamicin (50 µg/ml) in the tissue digestion medium and the initial medium for the culture of PMEC freshly isolated from the porcine mammary gland,

because gentamicin is known to induce apoptosis (programmed cell death) of fibroblasts (Servais et al., 2005), but MEC are highly resistant to gentamicin-induced apoptosis (Murtagh et al., 2004). This method helped to get eliminate fibroblasts from the PMEC preparation.

For cryopreservation, 1×10^6 cells were resuspended in 1 ml of a sterile solution (90% FBS and 10% DMSO) and 1 ml of this solution was transferred to a screw top cryovial (Ma et al., 2015). The cryovials were placed in a freezing chamber, filled with isopropanol (Cat # 270490, Sigma-Aldrich, city and state), and stored at -80°C for 16 h. The cryovials were then immediately transferred to a liquid-nitrogen chamber for long-term storage.

GROWTH OF PMEC

Approximately 1×10^4 PMEC cells were seeded and cultured in DMEM/F12 growth medium, as described previously. At 24, 48, 72, 96, and 120 h of culture, cell numbers were determined using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Cat # CGD1-1KT, Sigma- Aldrich), as we described previously (Meininger and Wu 2002).

To determine effects of cryopreservation on cell growth, PMEC were thawed after they were cryopreserved at passages 3 or 15. The thawing procedures were the same as we described previously for endothelial cells (Wu and Meininger, 1993) and enterocytes (Haynes et al., 2009). Briefly, one cryovial containing the frozen PMEC cells was removed from a liquid nitrogen tank and immediately placed in a 37°C water bath.

After the cryovial was swirled gently for 45 sec, it was placed in a laminar flow hood, after the outside of the cryovial was wiped with 70% ethanol. The thawed cell solution was transferred to a 15-ml centrifuge tube, to which 10 ml of complete DMEM-F12 medium (at 25°C) was added slowly.

The centrifuge tube was centrifuged at $200 \times g$ for 5 min at 25°C. Inside the laminar flow hood, the supernatant fluid was aseptically aspirated, and the cell pellet was resuspend gently in 10 ml DMEM-F12 medium in preparation for cell culture. The growth or proliferation of thawed PMEC was determined as described previously.

DIFFERENTIATION OF PMEC IN RESPONSE TO PROLACTIN

To determine effects of prolactin on cell morphology, PMEC (5×10^4 cells) were seeded on a Lab-Tek II chamber slide (Cat # 154526, Nalge Nunc International, Rochester, NY) containing 1 ml DMEM/F12 containing 10% charcoal-stripped FBS, 10 µg/ml gentamycin, 0.5 µg/ml insulin, and 1 µg/ml hydrocortisone. Cells were cultured at 37°C until they reached 50% confluence and then the medium was changed and contained 0 to 2 ug/ml prolactin, but no EGF was added to the medium.

The concentrations of prolactin in the culture medium were chosen on the basis of previous studies with mouse MEC (Edwards et al., 1998). The medium was changed every 24 h. The process of differentiation was carried out over a period of 3 d.

OIL RED-O STAINING OF PROLACTIN-INDUCED DIFFERENTIATION OF PMEC

Differentiation of PMEC induced by prolactin, as described previously, was confirmed by Oil Red-O staining of lipid droplets. Cells were rinsed in PBS before fixing with 2% paraformaldehyde (containing 0.02% glutaraldehyde) (Cat # 158127, Sigma-Aldrich) for 10 min, and then incubated in propylene glycol for 5 min.

Five minutes after staining with 0.3% Oil Red-O solution (Cat # O-0625, Sigma-Aldrich), cells were washed sequentially with 85% propylene glycol and distilled water. The images were observed and captured using a phase contrast microscope (Model IX81, Olympus Microscope, Hicksville, NY).

DETECTION OF β-CASEIN mRNA

Total cellular RNA was extracted from cells in porcine milk or PMEC cells using Trizol according to the manufacturer's protocol (Invitrogen Life Technologies, Carlsbad, CA, USA). The isolated RNA was reverse-transcribed using the ThermoScript RT system (Cat # 11146-016, Invitrogen) for reverse-transcription real-time polymerase-chain reaction (RT-PCR), according to the manufacture's protocol. Primers of porcine *β-casein* (Gen bank accession no: X54974) were: forward 5'-TAGCCCATCCTTACACCGAG-3' reverse 5'-TAGCCCATCCTTACACCGAG-3'. Primers of the porcine β-actin were:

forward 5'-TCCACCGCAAATGCTTCTAG-3,

reverse 5'-TGCTGTCACCTTCACCGTTC-3'.

The amplification of the two genes required 30 cycles of the polymerase chain reaction. Each cycle was set as follows: 30s denaturation at 94°C, 45s annealing at 58°C and 45s extension at 72°C. PCR products were separated by 1.5% agarose gel electrophoresis and visualized after ethidium bromide staining (Ma et al., 2015).

DETECTION OF β-CASEIN PROTEIN RELEASED BY PMEC

The abundance of β-casein in the conditioned PMEC culture medium was determined by western blot analysis using an antiβ-casein monoclonal antibody (Cat # sc-30042, Santa Cruz Biotechnology, Santa Cruz, CA), as we described for other proteins (Ma et al., 2015).

Briefly, PMEC were cultured for 3 d in the presence or absence of prolactin as described previously. Cells were pelleted and lysed in cold lysis buffer using published methods (Lei et al., 2012).

Total protein (5 to 40 μg) was separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blotted with 3 % BSA in Tris-Tween buffered saline (TTBS; 20 mM Tris/150 mM NaCl, pH 7.5, and 0.1% Tween-20) for 2 h and then incubated with a primary antibody (1:1,000 dilution) against β-casein (Cat # sc-30042, Santa Cruz Technology) or β-tubulin (Cat # 2128, Cell Signaling) overnight at 4°C.

Thereafter, the membrane was washed three times with 1XTTBS. The membranes were incubated at 25°C for 2 h with a goat anti-rabbit secondary immunoglobulin (Cat # 7074S, Cell Signaling Technology, Boston, MA) at 1:5000 dilution. Finally, the membranes were washed three times with TTBS, followed by exposure to the Super

Signal West Dura Extended Duration Substrate according to the manufacturer's instructions (Pierce, Rockford, IL).

DETECTION OF α-LACTALBUMIN PROTEIN RELEASED BY PMEC

Western blot analysis of α-lactalbumin in the conditioned PMEC culture medium was performed as described previously for β-casein, except that primary antibodies (1:10,000 dilution) against α-lactalbumin (Cat # A10-128P, Bethyl Laboratories, Montgomery, TX) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cat # 2118, Cell Signaling, Boston, MA) were used.

IMMUNOFLUORESCENCE STAINING OF B-CASEIN CYTOKERATIN AND VIMENTIN

The PMEC were treated with or without prolactin for 3 d, as described previously. For immunofluorescence staining of β-casein, cells were washed with PBS containing 0.3% (vol/vol) Tween-20 (Cat # 166-2404, Bio-Rad) (0.3% Tween-20/PBS), and fixed in 2% paraformaldehyde in 0.02 M PBS.

Cells were then washed three times in 0.02 M PBS and then incubated with 1% Triton X-100 (Bio-rad:161-0407) in 0.02 M PBS. Cells were subsequently washed three times with 0.3% Tween-20/PBS, suspended in 1% BSA at 25°C, and incubated overnight at 4°C with a primary antibody [anti-mouse β-casein (Cat # FL-231, Santa Cruz Technology), anti-mouse cytoketratin-18 (Cat # 49779, Abcam, Cambridge, MA, USA), or anti-vimentin (Cat # 03-61013, American Research Products, Waltham, MA) at 1:200 dilution in buffer (1% BSA in 0.3% Tween-20/PBS).

The Mouse IgG monoclonal antibody (Cat # I-5381, Sigma-Aldrich) was used as the negative control. Slides were washed three times in 0.3% Tween-20/PBS, and then incubated with the goat anti-mouse IgG secondary immunoglobulin (Cat # A-11032, Invitrogen) for 2 h at 25°C.

Thereafter, each slide was washed three times for 10 min each with 0.3% Tween-20/PBS, followed by rinsing with water. Slides were overlaid with the Prolong Gold Anti-fade/DAPI mounting reagent (Cat # P36935, Molecular Probes, Eugene, Oregon), covered with a thin glass, and incubated at 37°C for 1 h. Images were acquired using the Stallion Digital Imaging workstation at Texas A&M University (College Station, TX).

WESTERN BLOT ANALYSIS OF VIMENTIN IN PORCINE MAMMARY TISSUE

To show that the antibody against vimentin worked for porcine cells, we performed western blot analysis of vimentin in porcine mammary tissue. The procedure was the same as that described previously for β-casein, except that a vimentin antibody (1:10,000 dilution) was used.

EVALUATION OF CELL GROWTH USING THE MTT ASSAY

Growth curves for PMEC were determined to ensure that cells used throughout our experiments were within the exponential growth phase. Cell proliferation was assessed by monitoring the conversion of MTT 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide to formazan. The reduction of MTT (Sigma- Aldrich: CGD1-1KT) is catalyzed by mitochondrial dehydrogenase enzymes and is therefore a measure of cell

viability (Mosmann et al., 1983). 1×10^4 cells/well were seeded in 6-well flat bottom culture plates in DMEM/F12 growth medium as described above. Cell numbers were observed each day using a microscope and growth of the viable cells was assessed on a daily basis using the MTT cell growth determination kit according to the manufacture's protocol. The growth curves were constructed by plotting absorbance against time.

STATISTICAL ANALYSIS

Data on cell growth were analyzed by one-way analysis of variance and the Student-Newman-Keuls multiple comparison test (Assaad et al., 2014). Probability values $\leq$ 0.05 were taken to indicate statistical significance.

CHAPTER III

RESULTS*

MORPHOLOGY OF PME CELLS

Primary cultures of PME cells were established by continuous removal of stromal cells and fibroblast and subculturing them for several passages. It was found that MEC were more resistant than fibroblasts to treatment with trypsin-EDTA when the cells were detached from their the culture dish. PMEC formed islands and when grown to confluence, the cells formed a monolayer and aggregate with the characteristic cobblestone morphology (Wellnitz and Kerr, et al., 2004) of epithelial cells grown on chamber slides (Fig. 1A).

PMEC formed dome-like structure which had lumen-like structures among the cells (Fig.1a). A part of the cells were round and flat, but a small number of cells were elongated (Fig 1a). Prolactin-treated cells were visualized using a phase contrast microscope (Model IX81; Olympus) and the morphology of the majority of the PMEC changed considerably and cell surfaces were stained with oil Red O which detected fat in drops of milk (Fig 1a).
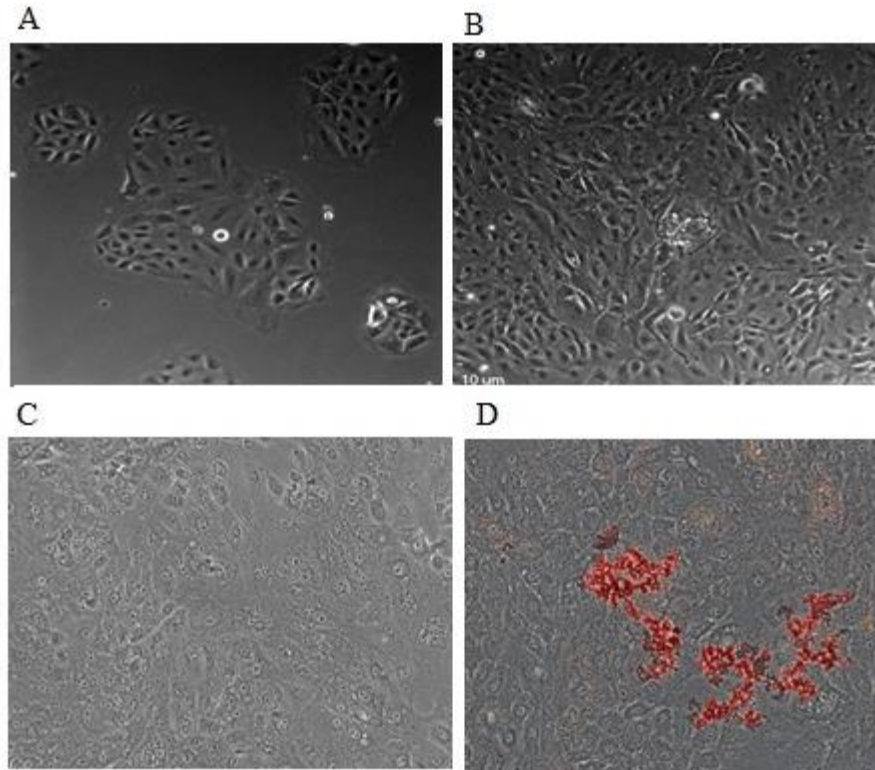
Figure 1a. Morphology of porcine mammary epithelial cells (PMEC) cultured with or without prolactin for 3 d. Cells at passage 15 were used for the experiment. (A) Porcine mammary epithelial cells without prolactin treatment had a cobblestone morphology. (B) Porcine mammary epithelial cells treated with 2 μg/mL prolactin contained white milk fat globules. (C) Oil Red-O staining of PMEC without prolactin treatment. (D) Oil Red-O staining of PMEC treated with 2 μg/mL prolactin, with the cells exhibiting red–white colored lipid droplets.
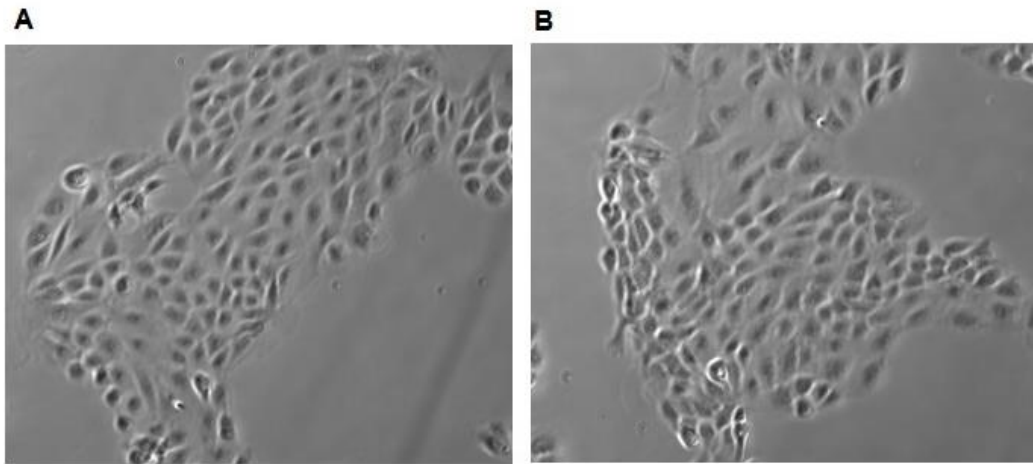
Figure 1b. The morphology of PMEC at two different passages. (A) passage 15 and (B) passage 3
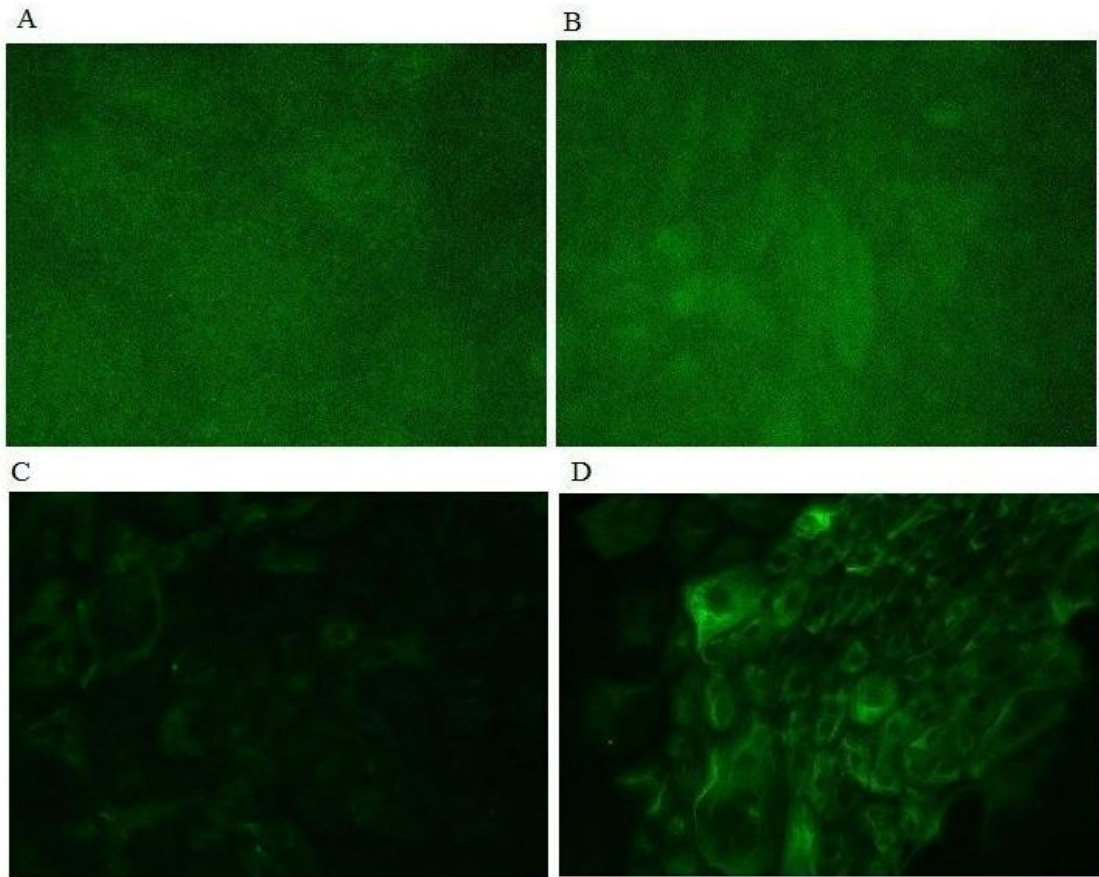
Figure 2. Immunofluorescence staining porcine PMEC for Vimentin (panels A and B) and cytokeratin-18 (panels C and D). Cells at passage 15 were used for the experiment. (A) The negative control for vimentin (PMEC incubated with the primary antibody against mouse IgG). (B) PMEC were negative for vimentin staining (PMEC incubated with the primary antibody against vimentin). (C) The negative control for cytokeratin-18 (PMEC incubated with the primary antibody against the mouse IgG). (D) The PMEC were positive for cytokeratin- 18 staining (PMEC incubated with the primary antibody against cytokeratin-18).
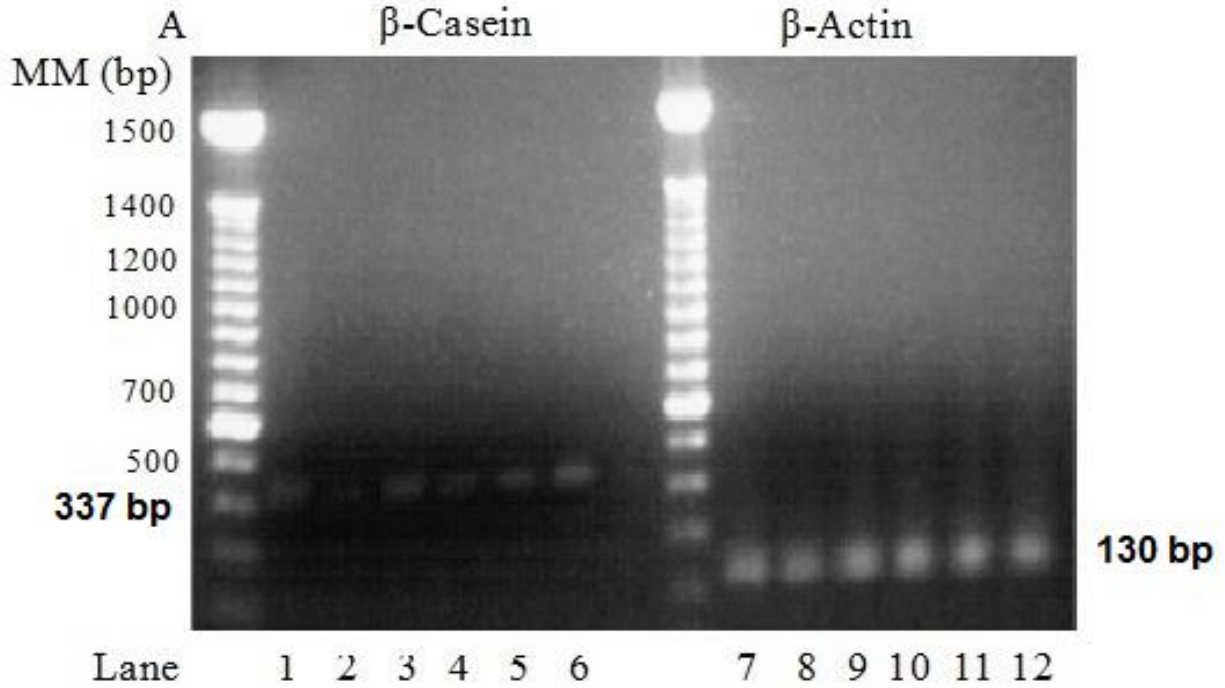
Figure 3. Expression of β-Casein mRNA and protein by the PMEC cultured with or without prolactin for 3 d. Cells at passage 15 were used for the experiment. (A) Messenger RNA levels for β-*casein* (lanes 1–6) and the housekeeping gene β-*actin* (lanes 7–12) in PMEC cultured with or without prolactin for 3 d. Labels for β-casein mRNA expression are as follows: lane 1: total RNA isolated from sow's milk (positive control); lane 2: PMEC without prolactin treatment; lanes 3 and 4: PMEC treated with 0.2 μg/mL prolactin; and lanes 5 and 6: PMEC treated with 2 μg/mL prolactin. Labels for β-actin mRNA expression are as follows: lane 7: total RNA isolated from sow's milk (positive control); lane 8: PMEC without prolactin treatment; lanes 9 and 10: PMEC treated with 0.2 μg/mL prolactin; lanes 11 and 12: PMEC treated with 2 μg/mL prolactin
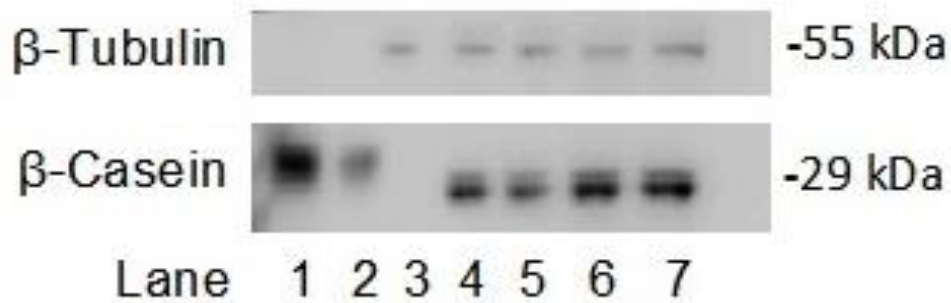
Figure 4a. β-Casein protein in PMEC cultured with or without prolactin for 3 d. lane 1: sow's milk at d 14 of lactation (40 μg milk protein; positive control); lane 2: sow's milk at d 14 of lactation (20 μg milk protein; positive control); lane 3: PMEC without prolactin treatment; lanes 4 and 5: PMEC treated with 0.2 μg/mL prolactin; and lanes 6 and 7: PMEC treated with 2 μg/mL prolactin. The duplicate lanes represent PMEC from 2 different culture plates of the same treatment. MM = molecular marker
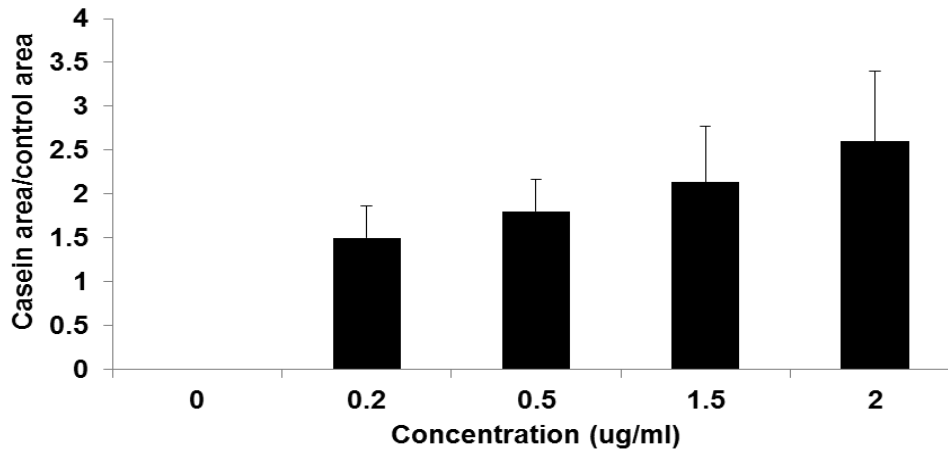
Figure 4b. Quantitative analysis of beta Casein expression compared with the absence of prolactin (lane 3: 0 μg/ml), addition of 0.2-2 μg/ml prolactin (lane 4-7) to culture medium for three days stimulated production of beta-casein by PMEC.
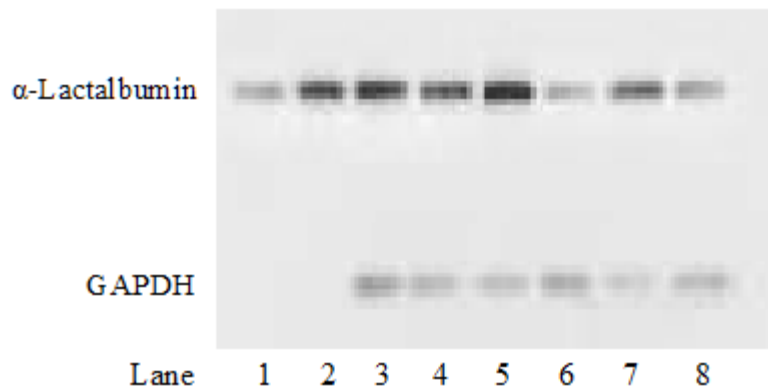
Figure 5. Expression of α-lactalbumin protein in the PMEC cultured with 0.2 or 2 µg/mL prolactin for 3 d. Cells at passage 15 were used for the experiment. Lane 1: sow's milk at d 14 of lactation (20 µg milk protein; positive control); lane 2: sow's milk at d 14 of lactation (40 µg milk protein; positive control); lanes 3 through 5: PMEC treated with 2 µg/mL prolactin; and lanes 6 through 8: PMEC treated with 0.2 µg/mL prolactin.
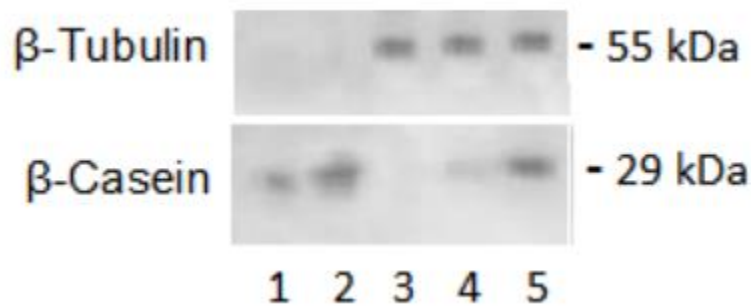
Figure 6. Western blot analysis of β-Casein and β-Tubulin proteins in cultured porcine mammary epithelial cells (PMEC). Lane 1: sow's milk (5 µg protein) at d 14 of lactation; Lane 2: sow's milk (10 µg protein) at d 14 of lactation; Lane 3: PMEC (passage 15) without prolactin treatment; Lane 4: PMEC (passage 15) treated with 0.2 µg/ml prolactin; Lane 5: PMEC (passage 15) treated with 2 µg/ml prolactin.
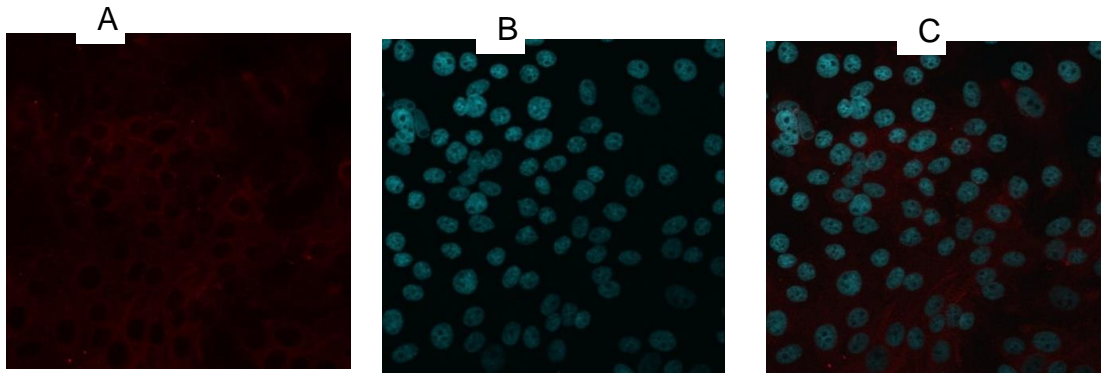
Figure 7. Immunofluorescence for β-Casein expression in PMEC. (A) Immunostaining PMEC with beta-casein antibody. (B) DAPI nuclear staining in PMEC. (C) Merged beta-casein and DAPI staining.
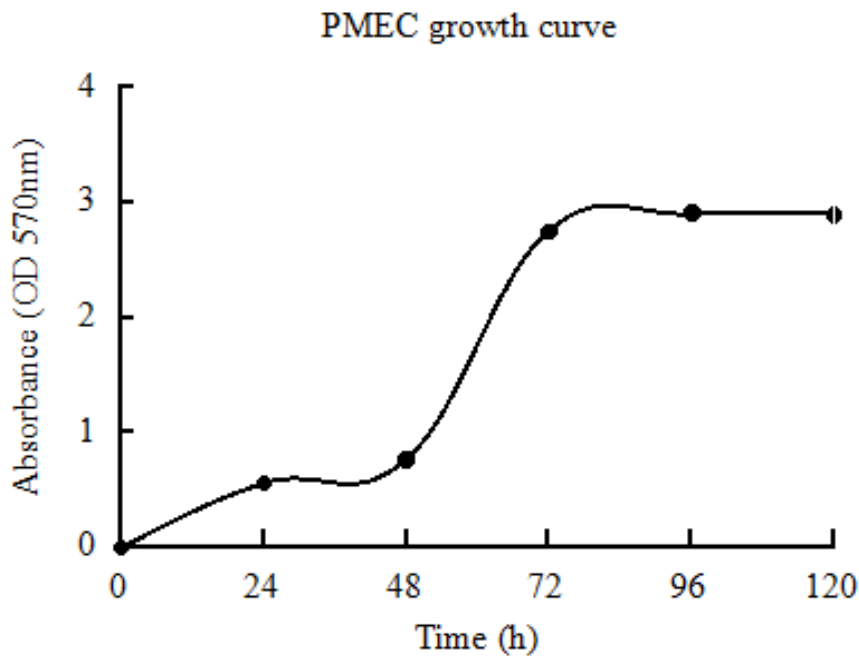
Figure 8. PMEC's growth curve assayed during a 120 h incubation period. Each time point represents the mean value of absorbance (n=3). PMEC growth was assessed by MTT and values plotted as absorbance of viable cells against time (0, 24, 48, 72, 96, and 120h) At stated time points, the cells were incubated with medium containing 1.0 mg/ml MTT at 37°C for 4 h. The absorbance of the solubilized crystals was detected using a microplate reader (Molecular Device Spectramax) at 570 nm, and the absorbance was directly proportional to viable cell numbers.

Table 1. Effects of cell passages and cryopreservation on porcine mammary epithelial cell (PMEC) growth

Cell Passage	Hours in culture				
	24	48	72	96	120
PMEC without cryopreservation					
3	10.4 (0.74)[c]	14.3 (1.49)[b]	51.0 (3.34)[a]	54.2 (3.72)[a]	54.0 (3.16)[a]
15	10.4 (0.68)[c]	14.2 (1.35)[b]	50.7 (3.02)[a]	54.0 (3.56)[a]	53.8 (3.30)[a]
PMEC with cryopreservation					
3	10.1 (0.62)[c]	14.0 (1.40)[b]	50.2 (3.15)[a]	53.5 (3.64)[a]	53.6 (3.44)[a]
15	10.2 (0.65)[c]	14.1 (1.46)[b]	50.5 (3.27)[a]	53.7 (3.88)[a]	53.9 (3.76)[a]

[a–c] Within a row, means sharing different superscript letters differ ($P < 0.05$). 1The number of PMEC was 10.0×10^3 at the time of seeding onto culture plates (0 h) for all treatment groups. Values, expressed as $\times 10^3$ cells/ well, are means (SEM); n = 5 independent experiments. See the text for details of cryopreservation of PMEC at passages 3 or 15.

RESULTS AND DISCUSSION

We successfully isolated PMEC from a 9-mo-old non-pregnant and non-lactating gilt using collagenase and cultured the cells in DMEM/F12 medium for at least 15 passages in a flat-bottom plate (Fig. 1b). The morphology of PMEC was similar between passages 3 and 15. These cells had a doubling time of about 24 h when grown in DMEM/F12 medium (Table 1). They grew as a monolayer with a cobblestone, epithelial-like morphology and formed islands of monolayer aggregates (Fig. 1b), which are the typical characteristics of MEC (Hu et al., 2009; Huynh et al., 1991). In addition, PMEC expressed cytokeratin-18, a specific marker of the epithelial cell lineage (Bartek et al., 1985; Hu et al., 2009), as determined by immunocytochemistry (Fig. 2). Vimentin, a specific marker for fibroblasts (Goodpaster et al., 2008), was not detected in the PMEC (Fig. 2). As a positive control, the vimentin protein was readily detected in porcine mammary tissue that contained fibroblasts. Cryopreservation of PMEC in 90% FBS and 10% DMSO allowed these cells to be stored in liquid nitrogen for a prolonged period of time. The PMEC were viable after thawing and grew similarly to those without cryopreservation (Table 1) and the frozen and thawed PMEC produced β-casein.

Because of successful cryopreservation, there is no need to sacrifice another gilt to obtain PMEC. It should be borne in mind that our stable nonimmortalized PMEC line was derived from primary PMEC. The β-casein gene is expressed only in MEC and is responsible for the production of β-casein protein by the lactating mammary gland (Pantschenko et al., 2000). Therefore, PMEC were evaluated for expression of its

hallmark gene, β-casein. We found that the PMEC expressed the gene for β-casein (Fig. 3). Porcine MEC cultured in the absence of prolactin did not synthesize or secrete the β-casein protein (Fig. 3). As a positive control, β-casein was readily detected in sow's milk (Fig. 3). Similarly, α-lactalbumin expression, another marker of MEC, was detected in sow's milk and prolactin-treated PMEC (Fig. 6).

PMEC without prolactin treatment did not produce α-lactalbumin. Large amounts of proteins and other substances in sow's milk may inhibit migration of β-casein on agarose gels in western blot analysis. This may explain why β-casein in sow's milk containing 20 or 40 μg of total protein did not migrate the same as β-casein produced by cultured PMEC shown (Fig. 3). When the amount of total milk protein used for the western blotting was reduced from 40 to 5 and 10 μg, migration of β-casein in sow's milk and PMEC culture medium was similar.

PMEC were sensitive to induction by prolactin. The MEC of the lactating mammary gland synthesize large amounts of triglycerides (Manjarin et al., 2012). Differentiated MEC have the same capability in culture, as indicated by the presence of neutral triglycerides and lipids detected using lysochrome diazo dye Oil Red-O (Hu et al., 2009). Therefore, to determine the ability of PMEC to differentiate in response to prolactin, the cells were cultured in serum-free DMEM/F12 medium supplemented with or without prolactin. Large Oil Red-O–stained lipid droplets in PMEC appeared after a 3-d exposure to prolactin (Fig. 1). The milk fat droplets in the cells were clearly indicated by the red staining. Additionally, in the presence of prolactin, PMEC synthesized β-casein (Fig. 1) and α-lactalbumin (Fig. 5) in a dose-dependent manner, as

reported for lactating mammary glands of mammals (Simpson et al., 1998; Shennan and Peaker, 2000). These results indicate that our PMEC are highly responsive to physiological stimuli that induce synthesis of milk proteins such as β-casein and α-lactalbumin. Of note, PMEC at passage 15 were used for these experiments, and the cells maintained their morphology, as well as their potential for differentiation and expression of β-casein mRNA and protein.

To our knowledge, this is the first report of the establishment of PMEC from nonpregnant and non-lactating female pigs. We successfully isolated, cultured, and characterized PMEC by: (a) determination of cell morphology with the use of light microscopy, and (b) measurement of mRNAs and/or protein abundances for milk proteins and epithelial cell specific markers by using RT-PCR, western blotting, and immunofluorescence histochemistry.

Initial characterization focused on growth morphology and expression of the milk-specific protein, beta-casein, by cultured PMEC and then studies were extended to evaluate differentiation of the PMEC. The availability of PMEC will provide a useful cell model to understand how metabolism, growth and differentiation of mammary cells are regulated by amino acids and other factors. Subsequent studies may focus on cellular and molecular regulation of milk synthesis in mammary glands of the sow.

As reported in previous studies, researchers have been exploring two basic methods to culture MEC *in vitro*, either on extracellular matrices (collagen substratum) or directly on the plastic cell culture dishes. Here, we used a collagenase digestion method for isolating PMEC and applied the direct plating method to culture PMEC. In our

experiments using the DMEM/F12 medium, we observed a similar morphology of PMEC to that reported for other types of MEC by Zheng et al., (2010). Cobblestone appearance (Fig 1 b) of the monolayer of PMEC on plastic was apparent when cells confluence was between 20-60%. This characteristic feature of MEC also was visible in bovine MEC as observed by Zhao et al., (2010). The cobblestone morphological structure diminishes as cells reach a higher confluence, since the individual islands merged together. Many types of MEC cell lines form dome-like structures when cultured on plastic and formation of dome-like structures was associated with accumulation of fluid in the epithelial cells grown on plastic or glass implements (Pickett et al., 1975).

At very low confluence (20-40%), PMEC showed dome-like structures that appeared like a nipple and with time the cells formed a lumen-like structure. We also observed that PMEC formed an elongated dome-like elongated structure when cell confluency was over 50% (Fig 1a).

The major milk protein genes are defined as mammary-specific and developmentally-regulated. Secretion of milk proteins such as casein (a major milk protein) and WAP, as well as milk fat, is considered a characteristic feature of differentiated MEC (Aoki N et al., 2006). All these characteristics of MEC were observed in our PMEC cells.

There are many reports that bovine MEC secrete milk when cultured on collagen matrix in serum-free medium (Talhouk et al. 1990; Gibson et al., 1991; Ahn et al., 1995). In contrast to the previous reports, the bovine MEC maintained sensitive to lactogenic hormones, and synthesized and secreted milk proteins even when cultured on plastic

dishes without a collagen matrix (Li et al., 2009) In our experiment, PMEC were grown on a nonmatrix environment, and these cells differentiated and expressed beta-casein in the presence of prolactin.

Our findings have important implications for the study of lactation biology, as expression of the beta-casein gene is a specific indicator of differentiation of MEC. When cultured on a plastic dish, our PMEC readily synthesized fat in response to prolactin (Fig. 1B). Detection of lipid droplets in the cytoplasm of PMEC by Oil-red O staining Fig. (1D) suggests that the PMEC differentiated into a more mature cell type in culture.

Secretion of lactose and beta-casein secretion by PMEC into culture medium is induced by prolactin (Levine and Stockdale, 1985). Beta-casein protein was not found in fetal calf serum or in the basal culture medium. Thus, the presence of beta-casein in PMEC-conditioned culture medium indicated functional differentiation of the PMEC.

As indicated in previous studies, several growth factors are associated with differentiation of PMEC. Prolactin is one of the factors that stimulates proliferation and differentiation of ME during pregnancy. This hormone is also essential for the secretion of milk by inducing expression of milk proteins.

Expression of beta-casein is considered to be a hallmark response of the MEC to hormonal induction (e.g., prolactin) (Hu et al., 2009). Western blot analysis indicated an increase in beta casein mRNA in PMEC in response to prolactin supplementation (Fig. 4a). Compared with the absence of prolactin, addition of 0.2-2 µg/ml prolactin to culture

medium for 3 days stimulated production of beta-casein by PMEC in a dose-dependent manner when normalized against tubulin as an internal control (Fig 4b).

Amplification of the transcripts of beta-casein by RT-PCR in PMECs in the present investigation suggested that the cells were functionally differentiated. Similar findings were reported for lactating bovine MEC (Kumura et al., 2001). Therefore, the presence of beta-casein detected at the mRNA level by RT-PCR (Fig 3) and the protein level by western blotting (Fig 3B) in PMEC indicates that these cells underwent differentiation so that they expressed lactation-specific proteins (beta-casein) in the presence of an exogenous prolactin. Meanwhile, we found that beta-casein localized to the cytoplasm of PMEC by immunofluorescence analysis (Fig 7).

We further determined expression of cytoskeleton protein markers for epithelial cells using the immunofluorescence technique. Cytokeratins are the keratin-containing intermediate filaments found in the intra-cytoplasmic cytoskeleton of epithelial cells and are important in defining the cell phenotype (Bartek J et al., 1985). The cytokeratin filaments appear as interconnected bundles in the cytoplasm. This cytoskeleton network is more dense around the nucleus, cytoplasmic vesicles and in the periphery of the cell where the filaments run parallel to the cell surface, which after several subcultures may decrease in the area surrounding the nucleus (Cifrain et al., 1994). Cytokeratin-18 is often used together with keratin 8 and keratin 19 to identify cells of epithelial origin from hematopoietic cells (Allard et al., 2004) and cytokeratin-18 is normally associated with simple epithelia and all luminal epithelial cells of the human mammary gland (Woelfle et al., 2004). Results of our immunocytochemial analysis showed that PMEC

were of the epithelial origin and stained positive for cytokeratin-18 (Fig 2D). Next, we examined the production of beta casein in PMECs by imunocytochemistry for PMEC cells grown on the plastic substratum in the presence and absence of prolactin (PRL). Positive immunostaining for beta-casein in PMECs was consistent with results from caprine (Pantschenko et al., 2000, Lee et al., 1985) and mouserry et al., 1987) MEC.

Lipid synthesis is a characteristic of differentiated MEC (NEEDS A REFRENCE). Milk fat droplets are secreted from MECs by a budding process in which droplets of triglyceride formed in the cytoplasm are gradually enveloped by a layer of apical plasma membrane called milk fat globule membrane (MFGM) (NEEDS A REFRENCE). Lipid accumulation measured by oil-red staining is a marker of mammary cell differentiation (Bancroft et al., 1984). We performed lipid staining using the Oil Red method and identified fat globules, which further strengthend the evidence that PMEC differentiated in response to prolactin induction (Fig 3). g 2B).

The growth curve shown in Fig 8 shows that the proliferation of the porcine PMEC doubled within 48-72h when grown on flat bottom plastic cell culture dishes in DMEM/F12 medium with supplements stated previously. The PMEC grew faster from day 3 to day 4 and cell growth extended around the plastic substratum byr day 5, but did not change in numbers   between days 4 and 5 when dead cells were observed floating in the medium.

Cell growth characteristic was carried out using cells in passage 8 and they were stored in liquid nitrogen in 95% fetal calf serum (FCS) and 5% of DMSO. These results are similar to those observed previously (Pantschenko et al 2000; Zavizion et al 1996).

Our results showed that frozen PMECs maintained normal growth characteristics upon thawing which indicated that 95% FCS and 5% of DMSO was optimal for cryopreservation of the PMEC.

CHAPTER IV

SUMMARY AND CONCLUSION

We established a stable non-immortalized PMEC line for laboratory research from non-lactating porcine mammary glands. These PMEC will be a useful model for future studies of porcine mammary gland development and differentiation, and as a prospecting screening system to study effects of nutrients on expression of milk proteins The basic characteristics of the PMEC were: 1) their expression of markers for the epithelial cell lineage; 2) their responsiveness to prolactin for induction of differentiation and production of β-casein and α-lactalbumin; 3) maintenance of cell morphology and the potential for differentiation after at least 15 passages in culture; 4) their expression of cytokeratin-18 as a marker of differentiated cells; and 5) their ability to synthesize lipids as a characteristic of differentiated mammary epithelial cells. Thus, our PMEC provides a useful tool for future studies of cellular and molecular mechanisms responsible for nutritional and hormonal regulation of lactation in sows.

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