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Establishment and characterization of a spontaneously immortalized porcine mammary epithelial cell line

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Abstract

We have established a spontaneously immortalized porcine mammary epithelial cell line (SI-PMEC) from the mammary gland of a lactating sow and maintained it long-term in culture by continuous subculturing. SI-PMEC cells were maintained for more than 8 months (70 passages) in DMEM/F12 medium supplemented with 10% fetal calf serum, insulin, and hydrocortisone without obvious signs of senescence. When grown at low density on a plastic substratum, SI-PMEC cells formed islands, and when grown to confluency, the cells formed a monolayer and aggregated with the characteristic cobblestone morphology of epithelial cells. The subcultured SI-PMEC cells appeared to proliferate without changes in morphology or growth pattern, with an estimated population doubling time of 20–22 h. With increasing density, SI-PMEC cells organized into lumen-like structures with elongated cells at the margins. SI-PMEC cells from stocks frozen at Passage 30 were subcultured up to 20 times without changes in cell viability, proliferation rate, or morphology. Furthermore, SI-PMEC cells remained immunopositive to an antibody against cytokeratin AE3 and immunonegative to an antibody against a human fibroblast antigen. The SI-PMEC cells could form functional structures resembling ducts, lateral-buds, and alveoli in a Matrigel matrix-dependent manner *in vitro*. When grown on the Matrigel and stimulated by prolactin, the cells differentiated and formed mammary gland structures and strongly expressed transcripts encoding the milk proteins α-lactalbumin, β-casein, and β-lactoglobulin. Our results indicate that the SI-PMEC cell line can be subpassaged many times and still form functional differentiated secretory structures. To our knowledge, this is the first report of an immortalized mammary epithelial cell line from pig.

Keywords: Pig; Mammary gland; Epithelial cell; Immortalized

1. Introduction

A mammary epithelial cell line retaining mammary gland-specific functions can reasonably be used as a model to study the development, differentiation and involution of mammary glands as well as an expression/selection system for producing protein pharmaceuticals. Transgene expression of complex proteins in milk has several advantages over prokaryotic and yeast expression systems (Zhang et al., 1998). For example, 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 milk yields from mice are very low. In contrast, cows and goats are good candidates for exogenous protein production because of high milk production (Janne et al., 1998; Wheeler and Walters, 2001; Niemann and Kues, 2003). The pig is an alternative candidate; its advantages include a short gestation period, large litter sizes 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: SI-PMEC, spontaneously immortalized porcine mammary epithelial cell line; RT-PCR, reverse transcription-polymerase chain reaction; α-La, α-lactalbumin; β-ca, β-casein; β-Lg, β-lactoglobulin.

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The main barrier to produce transgenic livestock is inadequate transgene expression. Thus, an *in vitro* screening system that identifies superior transgenes prior to transfer could improve transgenic animal production. To increase transgene product recovery from transgenic pig milk, it is desirable to use a fully functional and transfectable mammary epithelial cell line for rapid screening. A number of immortal mammary epithelial cell lines of human (Madsen et al., 1992), mouse (Danielson et al., 1984; Kittrell et al., 1992), cow (Gibson et al., 1991; Huynh et al., 1991; German and Barash, 2002; Rose et al., 2002), sheep (Düchler et al., 1998; Ilan et al., 1998) and goat (Pantschenko et al., 2000) have been established. Furthermore, Kumura et al. (2001) as well as our laboratory have isolated porcine primary mammary epithelial cells and evaluated mammary gland-specific functions in isolated cells. To our knowledge, however, there are no reports describing the establishment and characterization of a permanent porcine mammary epithelial cell line that proliferates indefinitely when appropriate medium and space are provided.

In the present study, we describe the establishment of a spontaneously immortalized porcine mammary epithelial cell line, SI-PMEC, and the characterization of its growth behavior, differentiated morphology and hormone responsiveness.

2. Materials and methods

2.1. Cell preparation and culture

Porcine mammary epithelial cells were isolated from the mammary gland of a lactating Landrace pig (Sun et al., 2005) 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 1X Dulbecco's Phosphate Buffered Saline (D-PBS; GIBCO, Rockville, MD, USA) containing 0.1% collagenase A (Type III; Sigma, St. Louis, MO, USA), 0.05% hyaluronidase (Sigma), 2.4 U/ml dispase II (GIBCO) and 1X PSN antibiotics (50 μg/ml penicillin G, 50 μg/ml streptomycin sulfate, and 10 μg/ml neomycin sulfate; GIBCO). After filtration through stainless steel meshes (80-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 cells were cultured in basal medium, which comprised DMEM/F12 (GIBCO) containing 10% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel), insulin (10 μg/ml; Sigma), hydrocortisone (1 μg/ml; Sigma), and 1X PSN antibiotics. Approximately 5×10^6 cells were seeded and cultured on 9 cm plastic dishes at 37 °C in a humidified atmosphere containing 5% CO_2. The attached cells were washed several times with D-PBS containing 1X PSN antibiotics after 24 h cultivation, and then fresh basal medium was added for continuous culturing.

2.2. Serial passage and establishing SI-PMEC

Cultured porcine primary mammary epithelial cells were seeded at a density of 5×10^5 cells in tissue flasks and maintained in basal medium every 4–6 days until subpassaged. Cell cultures were subpassaged when 80–90% confluent by removing the medium, washing the flask with D-PBS, treating with 0.25% trypsin-EDTA (GIBCO) and incubating at 37 °C until the cells detached from the flask. Then, the cells were centrifuged at 1000 rpm for 5 min and the supernatant was removed and the cell pellet resuspended in fresh basal medium for subculturing. The medium was renewed at intervals post-seeding.

2.3. Cell growth assays

The *in vitro* growth properties of SI-PMEC cells were assessed by their doubling time. The SI-PMEC cells at different passages were detached from a confluent culture by trypsin-EDTA dissociation and plated at 5×10^4 cells per 60-mm Petri dish. For each dish, cells were harvested every day for 6 days after inoculation. On the day of counting, SI-PMEC cells on the dish were dislodged by trypsin-EDTA and the cell clump was broken into a single cell suspension. An aliquot of cell suspension was diluted and assessed by trypan blue dye (Sigma) exclusion using a hemocytometer.

2.4. Assay of colony formation

Colony formation of SI-PMEC cells was assayed using a modified method reported by Faraldo et al. (2000). A concentration of 2% agar (Bacto-Agar; Difco Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was prepared in basal medium at 37 °C and added to 60-mm culture dishes. The agar was allowed to solidify and a suspension of SI-PMEC cells (5×10^4 cells/ml) was suspended in basal medium with 0.5% agar at 37 °C and liquid medium was added after 3 days inoculation and renewed every 3 days. To observe colony formation, cultures were fixed by adding 3.7% formaldehyde in PBS and colonies with more than 20 cells were counted using an inverted microscope at 100× objective lens magnification.

2.5. Chromosomal analysis

The cellular chromosomes were prepared using a method with slight modifications by Worton and Duff (1979). The SI-PMEC cells were grown in the presence of 0.1 μg/ml colcemid for 6 h at 37 °C. The cells were harvested by trypsinization, centrifugation and then carefully resuspended in prewarmed 0.075 M KCl (Merck, San Diego, CA, USA). After incubation for 30 min at 37 °C, the cells were pelleted again and fixed with a mixture of methanol:acetic acid (3:1) at 4 °C, and washed 3–4 times with the fixative solution. Finally, the cell suspension was dropped onto chilled glass slides, and stained with Giemsa (Sigma). The chromosomal number and modal karyotype of SI-PMEC cells were determined at Passage 20, 30, 50 and 70.

2.6. Immunohistochemistry

Monoclonal antibodies against cytokeratin AE3 (Type II, subfamily No. 1-8; Chemicon, Temecula, CA, USA) (Huynh et al., 1991) and human fibroblast antigen (Oncogene Research Products, San Diego, CA, USA) were used to characterize the epithelial cells. The SI-PMEC cells were plated in six-well plates (1×10^5 cells/well) for 24 h and then fixed in 95% ethanol for 1 min. Twenty microlitres 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 NovaRED Substrate Kit (Vector Laboratories, Inc.) and examined by light microscopy.

2.7. Culture with Matrigel

A six-well tissue culture plate was coated with BD Matrigel™ (5 μg/cm^3; BD Biosciences, St. Jose, CA, USA) and SI-PMEC cells (5×10^5 cells/well) in basal medium were then added directly. The cultures were incubated and the medium was changed every other day for up to 1 week and observed every day.

2.8. Determination of milk protein transcripts

For determining milk protein gene expression induced by Matrigel and lactogenic hormones, SI-PMEC cells cultured on Matrigel in basal medium

containing prolactin (5 μg/ml; Sigma) were used to prepare total cellular RNA for reverse transcription-polymerase chain reaction (RT-PCR). The PCR primers for amplification of α-lactalbumin, β-casein, and β-lactoglobulin transcripts 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 and Hsu, 2005). Total RNA was extracted from SI-PMEC (1×10^6 cells) using Trizol (GIBCO) in accordance with the manufacturer's instructions. Five micrograms of total RNA were reverse-transcribed using 2.5 μM oligo-dT primers (Promega, Madison, WI, USA), 1 mM of each dNTP (Promega), 20 U ribonuclease inhibitor (HT Biotechnology Ltd., Cambridge, England) and 5 U reverse transcriptase (RTase) (HT Biotechnology) in RT buffer (25 mM Tris–HCl, pH 8.3, 50 mM KCl, 2 mM DTT, and 5 mM $MgCl_2$, HT Biotechnology Ltd.) in a total volume of 20 μl at 39 °C for 60 min. For each PCR reaction, 3 μl RT product were 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 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.

3. Results

3.1. Morphology of SI-PMEC cells

Primary cultures of porcine mammary epithelial cells were established and were found to be more resistant than fibroblasts to treatment by trypsin and EDTA when the cells were detached from their substratum. Using this property, we established a spontaneously immortalized porcine mammary epithelial cell population by continuous removal of fibroblastic cells and subculturing. After 20 passages of subculturing/selection from the primary mammary epithelial cell culture, an immortalized cell line, named SI-PMEC, was established and continuously subcultured. The SI-PMEC cells formed islands and aggregated with the characteristic cobblestone morphology of epithelial cells when the cells were grown at low density on a plastic substratum (Fig. 1A). Fig. 1B shows that, in confluent cultures, SI-PMEC cells formed a monolayer with neighboring cells, and many blister-like structures were formed. The cells in post-confluent cultures formed a variety of different structures, including lumen-like structures with definite boundaries where extended islands had converged (Fig. 1C).

3.2. Growth characteristics of SI-PMEC cells

SI-PMEC cells subcultured at Passage 30, 50 or 70 proliferated without changes in morphology or growth pattern, and the population doubling time was estimated at 20–22 h (Fig. 2). The cells were grown for over 70 passages without

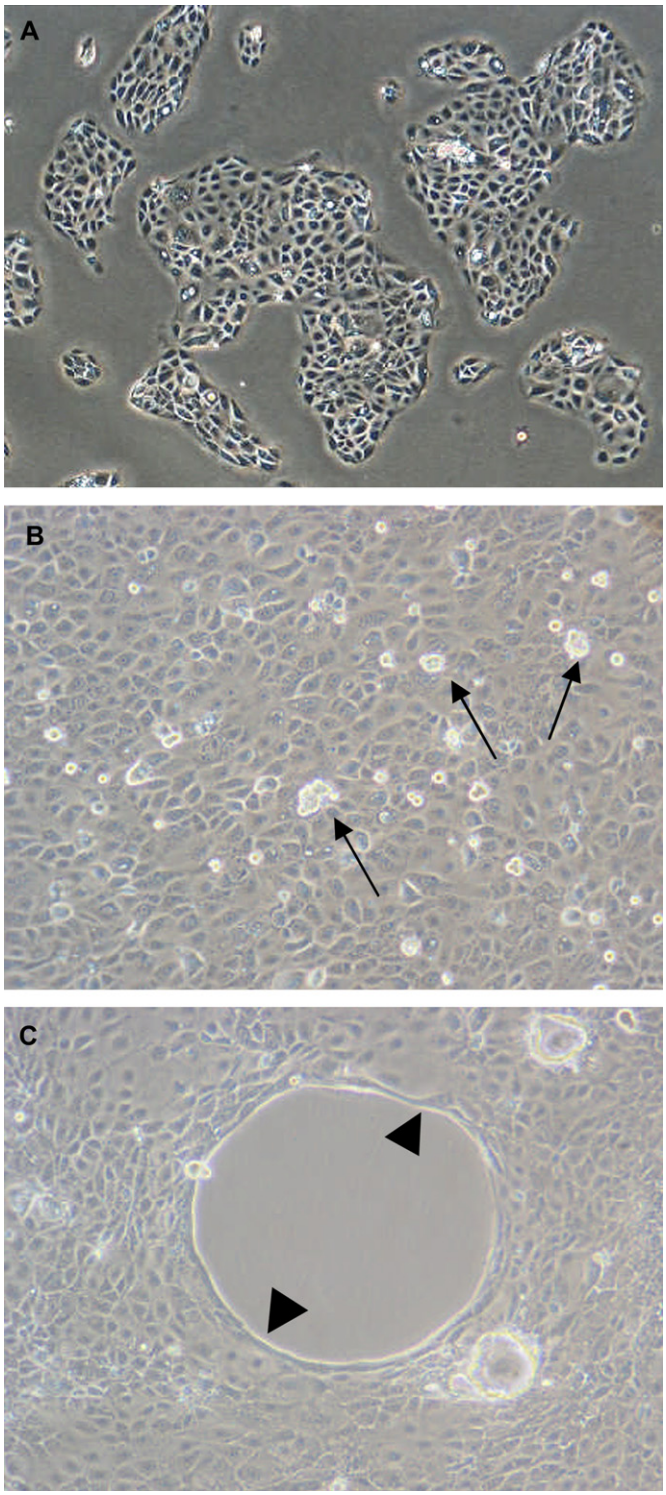

Fig. 1. Cytomorphologies of SI-PMEC cells cultured on a plastic substratum. SI-PMEC cells at Passage 50 were cultured in basal medium (DMEM/F12 medium supplemented with 10% fetal calf serum, 10 μg/ml insulin, and 1 μg/ml hydrocortisone) at low density on a plastic substratum. Islands with a cobblestone morphology characteristic of epithelial cells were observed (A; ×40). The Passage 50 cells were continuously grown to form a monolayer, and several blister-like structures (indicated by arrows) appeared in the confluent cultures (B; ×100). Three days after monolayer formation, the confluent culture formed lumen-like structures with elongated cells within the margins (arrowheads) (C; ×100).

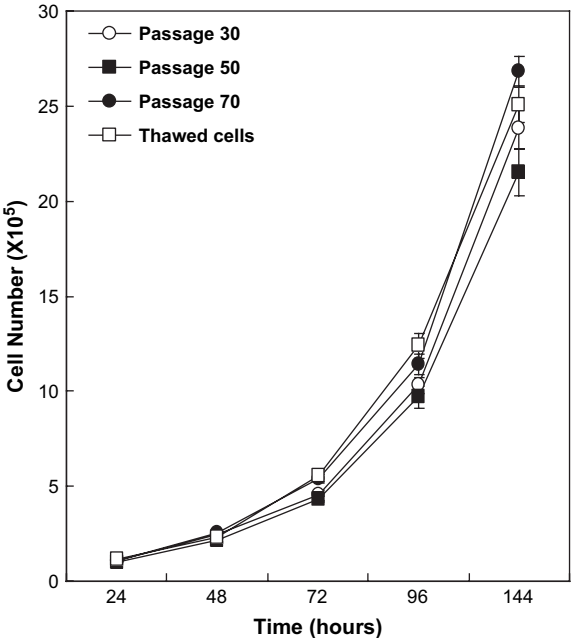

Fig. 2. Growth patterns of SI-PMEC cells. SI-PMEC cells at Passages 30, 50, 70 and cells frozen at Passage 30, stored in liquid nitrogen for 6 months and then cultured for 20 more passages, were seeded in 60-mm plates at a density of 5×10^4 cells/plate. For each culture, the cells were harvested every day for 6 days after inoculation. The cell number was determined using a hemocytometer. Each value represents the mean ± SD of three independent experiments.

changes in growth properties or viability (data not shown). SI-PMEC cells also maintained their growth characteristics without evidence of senescence after being stored at Passage 30 in liquid nitrogen for 6 months, thawed, and then cultured for 20 additional passages. In contrast, non-immortal early passage mammary epithelial cells became larger, grew more slowly and had more vacuoles after 15–20 passages in culture (Sun et al., 2005). The SI-PMEC cells did not develop a transformed phenotype, as overgrowth was not observed during routine subculturing. Further testing for transformation capacity indicated that SI-PMEC cells at Passage 70 did not form colonies after 3 weeks of culture in soft agar.

3.3. Immunohistochemical characterization of SI-PMEC cells

To further characterize the SI-PMEC cells, an 80% confluent monolayer was stained with cell type-specific primary antibodies followed by secondary antibodies. The SI-PMEC culture stained positively with anti-cytokeratin (antibody AE3), which is specific for epithelial cells (Fig. 3A), but showed no staining with anti-human fibroblast antigen marker, which recognizes the CD90 or Thy-1 surface antigen (Fig. 3B). These data suggest that SI-PMEC cells are not fibroblasts and retain epithelial characteristics.

3.4. Chromosome analysis

Metaphase chromosomes were prepared from SI-PMEC cells at Passages 20, 30, 50 and 70, and the chromosome

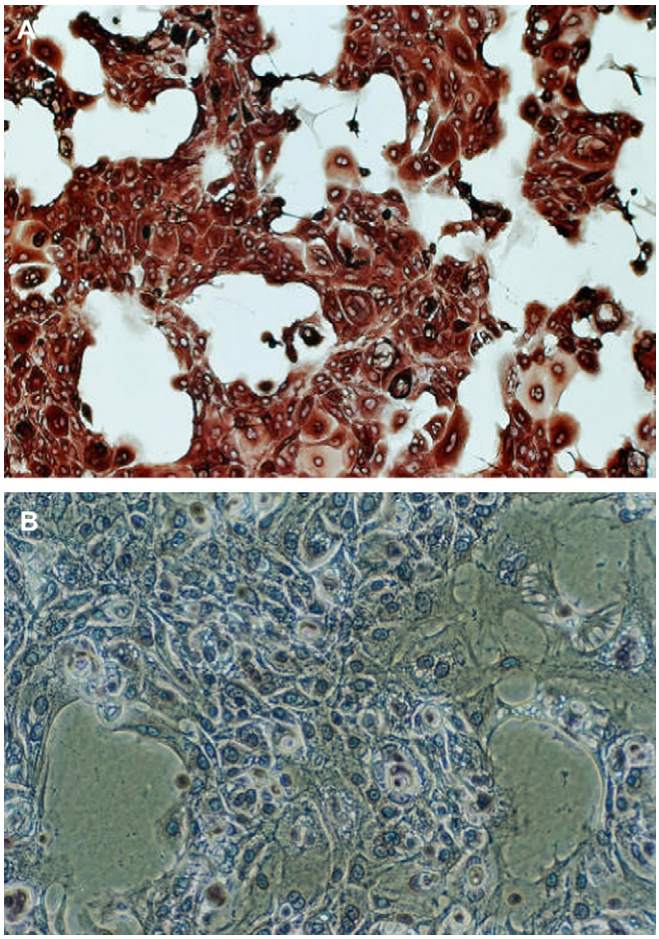

Fig. 3. Immunohistochemical analysis of SI-PMEC cells. SI-PMEC cells at Passage 50 remained immunopositive for anti-cytokeratin (antibody AE3), specific for luminal epithelial cells (A; ×200), and immunonegative for anti-fibroblast antigen (B; ×200).

number of approximately 50 nuclei at each passage was determined. The average number of chromosomes for SI-PMEC cells at Passages 20, 30, 50 and 70 was 37.9 ± 0.7, 37.6 ± 0.9, 37.2 ± 1.2 and 35.7 ± 1.4, respectively. The modal value of the cells at Passages 20, 30, 50 and 70 was 38, 38, 38 and 36, respectively.

3.5. Morphologic differentiation of SI-PMEC cells cultured with Matrigel

We evaluated the morphological differentiation of SI-PMEC cells into mammary duct-like structures when the cells were cultured with Matrigel. Matrigel is an extract from murine Engelbreth–Holm–Swarm tumor cells and contains laminin, collagen IV, entactin and a number of growth factors (McGuire and Seeds, 1989). Furthermore, Matrigel matrix has been shown to support differentiation and secretory activity in a variety of cultured mammary epithelial cells (Hurley et al., 1994; Rose et al., 2002; Wu et al., 2003). When dispersed SI-PMEC cell suspensions were cultured on Matrigel in basal medium, mammary gland-like structures were formed (Fig. 4). On the first day, the cells had already arranged

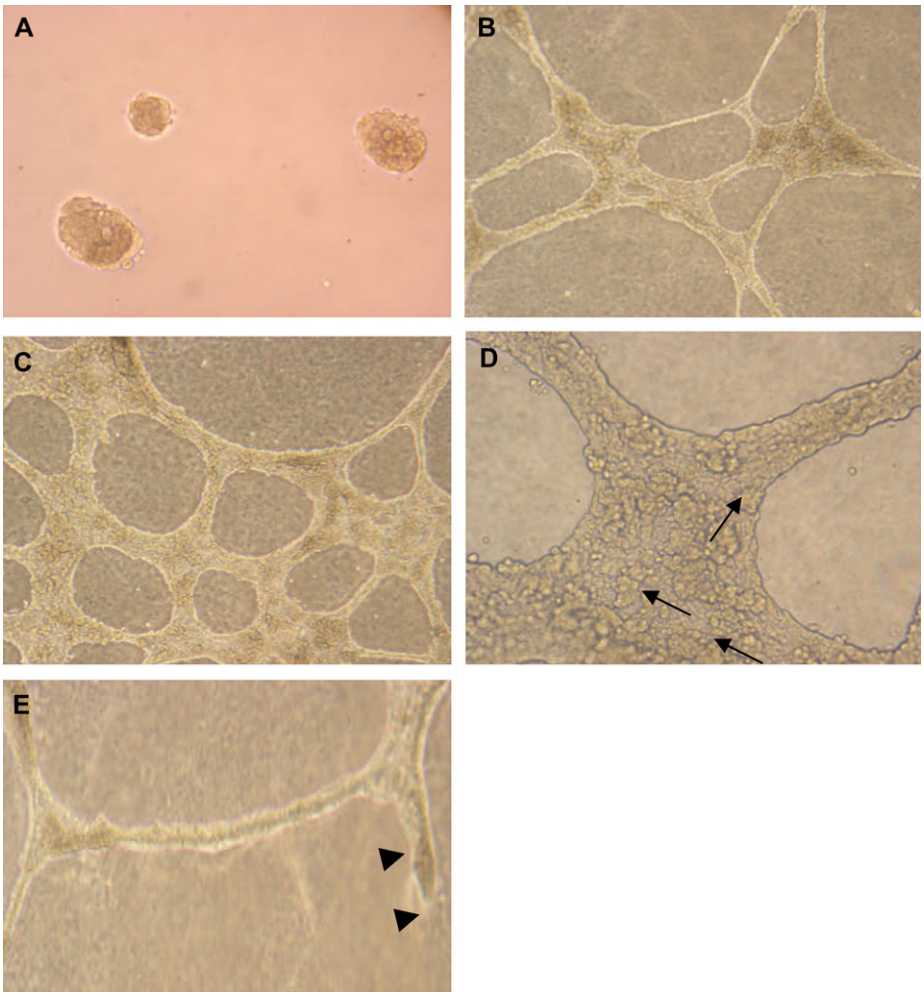

Fig. 4. Morphological differentiation of SI-PMEC cells cultured with Matrigel and prolactin supplementation. One day after SI-PMEC cells were plated on a Matrigel-coated dish containing prolactin in the basal medium, the cells aggregated to form mammosphere structures (A; ×100). Three days after SI-PMEC cell inoculation, the cells developed an elongated morphology and formed a simple three-dimensional duct-like structure (B; ×100). After culturing for five more days, the duct-like structures developed into a tubular network with lumens (C; ×100). The tubular network contained alveoli-like structures (indicated by arrowheads) (D; ×200), secretory end-buds and lateral-bud structures (arrows) (E; ×100).

themselves into a mammosphere structure (Fig. 4A). By day 3, the cells had an elongated morphology and formed a simple three-dimensional duct-like structure by migration and congregation (Fig. 4B). With continued incubation of the cultures, the duct-like structures were not only maintained but also continued to develop into a tubular network with lumens (Fig. 4C). The duct-like system was composed of clusters of alveoli-like structures (Fig. 4D), secretory end-buds and lateral-bud structures (Fig. 4E).

3.6. Gene expression of milk proteins

To determine the status of SI-PMEC cell differentiation, the levels of milk protein transcripts affected by substrata and lactogenic hormones in culture were compared. When SI-PMEC cells were grown on a plastic substratum, transcripts of α-lactalbumin, β-casein and β-lactoglobulin genes were undetectable. In contrast, when the cells were cultured on Matrigel supplemented with 5 μg/ml prolactin, α-lactalbumin, β-casein and β-lactoglobulin were strongly expressed (Fig. 5).

4. Discussion

A porcine mammary epithelial cell line would be useful for studies of species-specific protein expression and the proliferation of porcine mammary glands. However, most studies on porcine mammary glands have been limited to those using cultured explants (Jerry et al., 1989; Simpson et al., 1998) to date. Kumura et al. (2001) established a primary culture of mammary epithelial cells from pigs as a model system for evaluating milk protein expression. Our laboratory also cultured primary mammary epithelial cells from lactating sows and used the cells to express a heterologous protein (Sun et al., 2005). In this study, we describe the establishment and characterization of a porcine mammary epithelial cell line, named SI-PMEC, which arose from spontaneous immortalization after prolonged culture of our primary mammary epithelial cells (Sun et al., 2005). The established cell line has an advantage over primary cultures in that the cells retained consistent characteristics over the relatively long period of 70 passages. When grown on a plastic substratum, SI-PMEC cells displayed

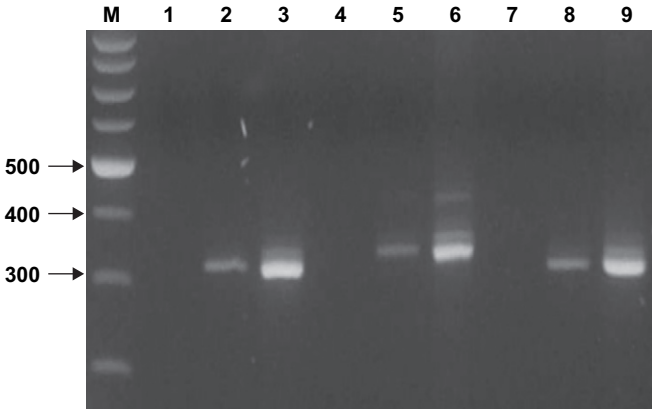

Fig. 5. Milk protein gene expression of SI-PMEC cells in the presence of prolactin and Matrigel. Passage 50 SI-PMEC cells were cultured in basal medium without prolactin supplementation on a plastic dish (lanes 1, 4 and 7), without prolactin supplementation on a Matrigel-coated dish (lanes 2, 5 and 8), or with 5 μg/ml prolactin on a Matrigel-coated dish (lanes 3, 6 and 9). The resulting amplified DNA fragments for α-lactalbumin, β-casein and β-lactoglobulin were 320, 341 and 328 bp, respectively. M, a 100-bp ladder; lanes 1–3, α-lactalbumin; lanes 4–6, β-casein; lanes 7–9, β-lactoglobulin.

rapid proliferation (Fig. 2), a cobblestone morphology typical of epithelial cells (Fig. 1), cytokeratin-positive staining, and contact-mediated differentiation in post-confluent cultures. When SI-PMEC cells were grown on dishes coated with Matrigel and stimulated by a lactogenic hormone, the cells differentiated into mammary gland-like secretory structures and strongly expressed the transcripts encoding the milk proteins.

The SI-PMEC cells showed a positive staining pattern with antibody AE3 against cytokeratin (Fig. 3A). This result provides direct evidence that the majority of SI-PMEC cells are luminal epithelial cells, as AE3 specifically stains the luminal epithelial cells of the mouse (Ilan et al., 1996) and sheep (Ilan et al., 1998) mammary gland. This identification is also supported by the ability of these cells to form highly developed alveoli- and duct-like structures that have luminal characteristics.

At Passages 20, 30 and 50, most SI-PMEC cells had a normal/near normal chromosome number consistent with that of cells of pig origin. However, there was evidence of chromosomal drift (pseudodiploidy), which is expected in long-term cell cultures (Pantschenko et al., 2000; Rose et al., 2002). After 70 passages, the proportion of heteroploid cells was significantly increased compared with early passages (data not shown). Therefore, we propose to limit the number of passages of SI-PMEC cells to less than 70 such that the cells might more closely maintain tissue-specific characteristics.

Formation of the differentiated structures by SI-PMEC cells depends on interaction with a Matrigel substratum, but is not absolutely dependent on the presence of prolactin in the culture medium (data not shown). As others have found for other cultured mammary epithelial cells, the Matrigel matrix supported differentiation and secretory activity of our SI-PMEC cells. The SI-PMEC cells grown in Matrigel-coated dishes exhibited a variety of different structures (Fig. 4). After approximately 5 days of incubation, elongation of the tubes and branching resulted in dense tubular networks (Fig. 4B and C); these phenomena have been also observed in other mammary gland epithelial cell lines (Danielson et al., 1984; Düchler et al., 1998; Pantschenko et al., 2000; German and Barash, 2002; Rose et al., 2002).

The origin of the proliferative capacity of SI-PMEC cells is not known but probably results from a random immortalizing event distinct from proliferation induced by mutagenic drug treatment (Kittrell et al., 1992) or viral transforming genes (Huynh et al., 1991; Gollahon and Shay, 1996). Several cell lines arose from spontaneous immortalization events (Pantschenko et al., 2000; German and Barash, 2002; Rose et al., 2002) also showed the morphological and functional characteristics of mammary gland as the case of SI-PMEC. Most importantly, SI-PMEC cells retained their ability to differentiate in culture and to recapitulate the basic *in vivo* structures of mammary glands, namely ducts, end buds, and multicellular alveoli-like structures (Fig. 4D and E).

Optimal expression of milk proteins by SI-PMEC cells requires not only stromal Matrigel, which supports differentiation by organizing the basement membrane and providing growth factors, but also stimulation by lactogenic hormones (Fig. 5). In a primary culture of mouse mammary epithelial cells, the amount of casein secreted into the medium containing insulin, cortisol and prolactin is 25–200 times greater than that secreted by cells cultured with insulin alone (Emerman and Pitelka, 1977). Wu et al. (2003) also demonstrated that prolactin and dexamethasone are strongly synergistic in inducing milk protein gene expression in the mouse mammary epithelial cell line NMuMG. Therefore, prolactin function is more important for the induction of milk protein gene transcription, translation, and secretion in mammary epithelial cells (Huynh et al., 1991; German and Barash, 2002; Wu et al., 2003).

In conclusion, this is the first study to report the establishment of an immortal mammary epithelial cell line, SI-PMEC, isolated from lactating porcine mammary gland. SI-PMEC cells may be a useful *in vitro* model for studies of porcine mammary development and differentiation. Moreover, the cells can also be used as an *in vitro* screening system to identify superior transgenes prior to transfer, thereby improving the efficiency of transgenic pig production.

Acknowledgements

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