Establishment and Characterization of a Yak Mammary Myoepithelial Cell Line (YMM)

[1]Mingchao Hao, [1,2]Ben Liu, [1,2]Jiangfeng Fan, [1]Mingliang Wang, [1]Peng Chen,
[1]Xudong Gao, [1]Hua Zhang and [1,2]Sijiu Yu
[1]Faculty of Veterinary Medicine, Gansu Agricultural University, Lanzhou, China
[2]Technology and Research Center of Gansu Province for Embryonic
Engineering of Bovine and Sheep and Goat, Lanzhou, China

Abstract: A Yak Mammary Myoepithelial cell line (YMM) from the mammary gland of a lactational yak was established and maintained long-term in culture by continuous subculturing. YMM cells were maintained for >6 months (40 passages) in DMEM/F12 medium supplemented with 10% fetal bovine serum without obvious signs of senescence. When grown on a plastic substratum, YMM cells showed a distinctive stellate morphology. Moreover, the cells formed a monolayer but did not form contiguous membrane to membrane contacts when grown to confluency. YMM cells appeared to proliferate without changes in morphology or growth pattern before passage 15 with an estimated population doubling time of 66-68 h. However, YMM cells changed to polygonal-like in morphology and their population doubling time reduced to approximately 59-61 h after stored in liquid nitrogen for 3 months and cultured for 5 more passages (passage 15). Furthermore, YMM cells remained immunopositive to antibodies against α-smooth muscle actin, cytokeratin7 and vimentin which suggested that the YMM cell line was myoepithelial origin. Noticeably, YMM cells appeared oxytocin responsiveness when supplemented oxytocin in medium and incubated for 30 min. Karyotyping analysis indicated that the cells maintained a normal diploid chromosome modal number of 2n = 60. In the opinion, the YMM cell line would become a useful model to study cell to cell communication and the mechanism in mammary growth and differentiation and cancerogenesis.

Key words: Yak, mammary gland, myoepithelial cell line, population, morphology, senescence

INTRODUCTION

Myoepithelial cells are located between the basement lamina and luminal cells and attached to the luminal cells by desmosomes and by hemidesmosomes to the basement lamina (Jolicoeur *et al.*, 2002). Myoepithelial cells express proteins typical for the contractile apparatus of smooth muscles cells, α-actin of Smooth Muscles (SMA), myosin heavy chains, α-actinin, vinculin and calponin (Sopel, 2010). Smooth muscle cells can be distinguished from myoepithelial cells on the basis of their intermediate filament content, the latter contains prekeratin intermediate filaments while the smooth muscle cells not (Franke *et al.*, 1980a, b).

The key role of myoepithelial cells is participating in the process of milk ejection. However, a great body of evidence has been accumulated suggesting the other roles of these cells in other important physiological processes such as the regulation of mammary gland growth, development and differentiation as well as the control of cancerogenesis (Gudjonsson *et al.*, 2002). Myoepithelial cells show a strong expression of receptor of EGF (epidermal growth factor) (Pilichowska *et al.*, 1997) and growth factors such as TGF (transforming growth factor) and FGF (fibroblast growth factor) (Rudland *et al.*, 1998). Gomma *et al.* (1997) described FGF as an important paracrine regulator of epithelial cell survival and growth in the normal human breast. Zhang *et al.* (1999) demonstrated that maspin exclusively expressed in myoepithelial cells and played a significant role in the morphogenesis, development and function of mammary gland. Myoepithelial cells can inhibit the growth of tumours and neoangiogenesis induce apoptosis and limit the mobility of cancer cells, so they are considered as natural suppressors of breast cancer (Sopel, 2010).

Intrinsically, the complex interaction of systemic and locally produced soluble factors and mesenchyme-derived cells makes it difficult to identify and dissect the

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Corresponding Author: Sijiu Yu, Faculty of Veterinary Medicine, Gansu Agricultural University, Lanzhou, China

contribution of different components which modulate the development, function and involution of the mammary gland epithelium (Pantschenko *et al.*, 2000b). The primary culture systems and establishment of cell lines are ideal methods to overcome these difficulties. A number of primary or immortal mammary myoepithelial cell lines of human (Peterson and van Deurs, 1988), rat (Soloff *et al.*, 1980; Warburton *et al.*, 1981), bovine (Zavizion *et al.*, 1992; 1996), caprine (Pantschenko *et al.*, 2000a) have been established. Approximately 14 million yaks are found in China, accounting for 90% of the total number of yaks in the world. Yaks play an important role in both livelihoods and natural ecosystems of the Qinghai-Tibetan plateau (Yang *et al.*, 2010). To the knowledge however, there are little reports describing the establishment and characterization of yak mammary myoepithelial cell line.

In the present study, researchers described the establishment of a yak mammary myoepithelial cell line, YMM and the characterization of its morphology, growth behavior, immunocytochemical properties, chromosomal analysis and oxytocin responsiveness.

MATERIALS AND METHODS

Mammary tissue dissociation and culture: Mammary tissue of a lactational yak was obtained from an abattoir of Xining city in Qinghai province. Fresh tissues were transported on ice in sterile Hank's (balanced salt solution) supplemented with 200 U mL^{-1} penicillin and 200 μg mL^{-1} streptomycin (Sigma-Aldrich). The tissues were washed with D-Hank's (balanced salt solution) solution for several times until the solution was pellucid and without milk, minced into small pieces (approximately 1 mm^3 in size) and washed three times in Phosphate Buffered Saline (PBS) containing antibiotics (200 U mL^{-1} penicillin, 200 μg mL^{-1} streptomycin) then the tissue pieces were dissociated by gentle agitation at 37°C for 6 h until the majority of parenchyma was dissociated in D-Hank's containing 250 U mL^{-1} collagenase (Type; Invitrogen), 150 U mL^{-1} hyaluronidase (Invitrogen) and antibiotics (200 U mL^{-1} penicillin, 200 μg mL^{-1} streptomycin).

The suspension was filtered through stainless steel 200-mesh sieves to remove undissociated tissues and debrises and then the cells were collected by centrifugation at 1000 rpm for 10 min and washed three times with PBS. Cell viability and number were determined by trypan blue exclusion. The isolated cells were plated and cultured onto 25 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) in DMEM/F12 (GIBCO,

Rockville, MD, USA) medium containing 10% Fetal Bovine Serum (FBS) (Hyclone) and antibiotics (100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin) at 37°C with saturated humidity and 5% CO_2.

Separation and enrichment of YMM cells: Cultured yak primary mammary cells were seeded at a density of 5×10^5 cells onto tissue flasks and subpassaged when 95% confluent by washing the flasks with PBS, detaching cells with 0.05% trypsin-0.04% EDTA (Sigma-Aldrich). Occasionally, researchers found a relatively pure myoepithelial-like cell population in one flask after subcultured for 3 passages, approximately 30% of the cells showed epithelial or fibroblastic morphology.

To isolate to homogeneity, we enriched the myoepithelial-like population by selective detachment method since, myoepithelial cells detach more readily than epithelial cells and more difficult than fibroblast. Selective detachment method was carried out by adding 1 mL trypsin-EDTA (0.05/0.04) to confluent 25 cm^2 flasks incubated at 37°C for 0.5, 2 and 5 min. At each time point, the supernatant was removed and fresh trypsin-EDTA was added. After 6 passages, the cells appeared >99% myoepithelial by morphology. Morphology of cultured cells was routinely evaluated with an inverted microscope with phase contrast (Olympus IX81, Japan) and photomicrographs were taken.

Growth characteristics of YMM cells: Growth curves and doubling time were determined by seeding 1×10^4 cells/well in 24 well tissue culture plates (Nunc). Media were changed every other day. Cell number was counted each day for triplicate wells using hemocytometer for 8 days. For each well, cells were harvested and counted 3 times. Growth curves were produced by plotting cell numbers versus time curves. Doubling times were estimated from the slope during the log phase.

The storage and recovery of YMM cells: The cells were propagated until confluent monolayer was reached. Fresh media was replaced 24 h prior to storage. The cell suspension from each flask was collected and total cell number was counted. The cells were then collected by centrifugation at 1000 rpm for 10 min. The pellet was resuspended in 4°C storage medium (70% DMEM/F12 medium, 20% FBS and 10% dimethyl sulfoxide) to obtain a concentration of 5×10^5 cells per milliliter. Approximately 1 mL aliquots were dispensed into 2 mL sterile freezing tubes (Nunc). The tubes were stored at 4°C for 1 h, -20°C for 1 h, respectively then -70°C overnight, at last

transferred into liquid nitrogen (-196°C). Cells were recovered after frozening for 3 months by shaking the tube in water at 37°C for 1 min.

Chromosomal analysis: The chromosomes of cells undergoing exponential growth were prepared using a method described by Swaminathan *et al.* (2010) with slight modifications. Cells grown on 25 cm^2 tissue culture flasks were treated with 1 µg mL^{-1} colchicine (Sigma) for 6 h, detached with trypsin-EDTA, washed centrifuged and then carefully resuspended in prewarmed hypotonic solution (0.075M Kcl) for 35 min at 37°C. Following these treatment, cells were fixed twice with ice-cold methanol and aceticacid mixture (volume 3:1) for 30 min then dropped onto chilled glass slides and stained with Giemsa (Sigma). The chromosomal number and modal karyotype of cells were determined at passage 10, 25 and 35.

Immunocytochemistry: The cells were cultured on sterilized glass coverslips in 6 well plates (1×10^5 cells well^{-1}) for 2 days. Cells were collected, washed in PBS and fixed in 4% paraformaldehyde for 90 min at 4°C. They were then treated with 0.5% Triton X-100 for 15 min and endogenous peroxidase was blocked by 0.3% H$_2$O$_2$ for 10 min. Pretreated cells were blocked for nonspecific binding with normal goat blocking serum of Histostain-plus kit (Invitrogen Zymed Laboratories, California, USA) then incubated with the primary antibodies: anti-cytokeratin7 (abcam, ab9021; UK) anti-vimentin (abcam, ab8978; UK) and anti-α-smooth muscle actin (abcam, ab7817; UK) for 2 h at room temperature. In control group, the primary antibody was instead of PBS. After that cells were incubated for 15 min with biotinylated goat anti-mouse IgG working solution (Invitrogen Zymed Laboratories, California, USA) and washed again in PBS before avidin-biotin peroxidase was applied.

Detection of the primary antibodies was performed with 3, 3'-Diaminobenzidine tetrahydrochloride substrate kit (DAB; Invitrogen Zymed Laboratories, California, USA). At last the cells were counterstained with haematoxylin.

Response to oxytocin: Researchers examined function differentiation of YMM cells by response to oxytocin. YMM or mammary epithelial cells were plating at 2×10^5 per well/3 mL media on 60 mm tissue culture dishes for approximately 5-7 days. During this time, cells formed fresh-confluent monolayers. The dishes were washed twice with DMEM/F-12 media and 3 mL of media with 50 µM oxytocin (Sigma) was added and then incubated at 37°C with saturated humidity and 5% CO$_2$. Changes in cell morphology were observed using an inverted microscope with phase contrast (Olympus IX81, Japan). The numbers of cells that contracted and/or detached from the dishes following treatment with oxytocin were recorded.

Sterility: Cultures of cells were routinely (every 10 passages) grown on coverslips and stained with Hoechst #33258 (Hoechst Ltd. Hounslow, Middlesex, UK) as described previously (Freshney, 1990) for evidence of mycoplasma contamination. Culture supernatants were tested for evidence of bacteria and fungus contamination with THIO media (Difco, Dickinson and Company, Franklin Lakes, NJ, USA). Researchers examined virus contamination by the sheep erythrocyte agglutination test.

RESULTS AND DISCUSSION

Morphology and growth characteristics of YMM cells: A yak mammary myoepithelial cell population was separated and purified by continuous removal of fibroblastic and epithelial cells from an early mixed culture of mammary cells. The myoepithelial cells were subcultured for >40 times since initiation and were designated as YMM cell line.

Morphologically, YMM cells had a special pattern and did not resemble the cobblestone morphology of yak mammary epithelial cells (Fig. 1f) nor the fusiform morphology of fibroblasts as well as their sizes were larger than epithelial cells and fibroblasts after attached to the culture dish. One day after plating, cells dispersed grew with a distinctive stellate morphology (Fig. 1a).

The cells then expanded in width and depth and extended out filopodia from the cell body at ends (Fig. 1b). After culturing for 7 days, cells reached confluency. Whereas cells grew in a random pattern which did not appear to form contiguous membrane to membrane contacts (Fig. 1c).

The shapes and sizes of YMM cells were changed after being stored in liquid nitrogen for 3 months, thawed and cultured for 5 more passages (passage 15) (Fig. 1d and e). YMM cells were maintained in continuous culture for >40 passages with no signs of senescence. Following subculture, YMM cells progressed through a lag phase within the 1st 2 days and entered into a log
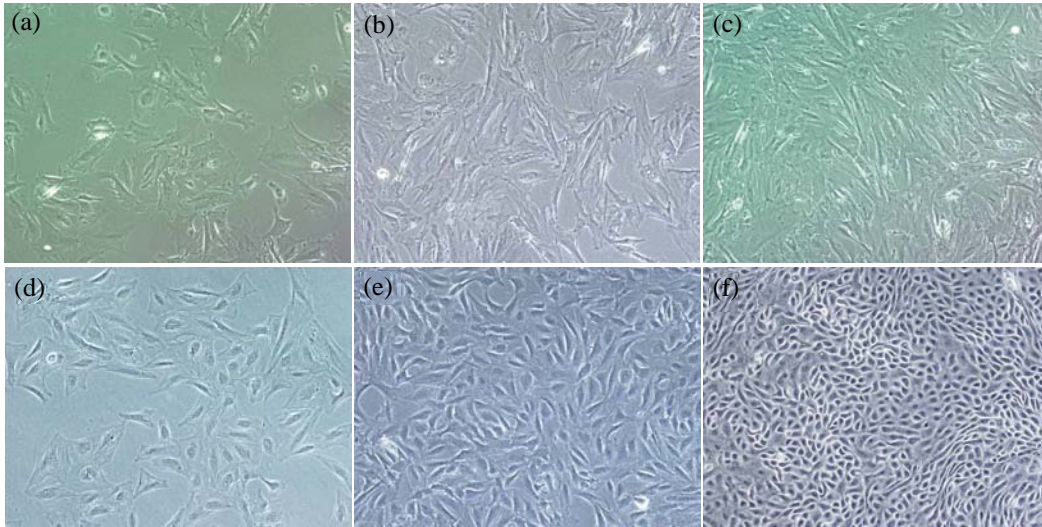
Fig. 1: a-e) Phase contrast photomicrographs of YMM cells and f) yak mammary epithelial cells; a) about 1 day after plating, YMM cells show a distinctive stellate morphology; b) preconfluent monolayer of YMM cells on 3 days; c) about 7 day confluent culture with spaces between cells; d) the preconfluent and e) confluent monolayers of YMM cells after shape changed (passage 15) and f) a confluent epithelial cells is shown for comparative purposes x200
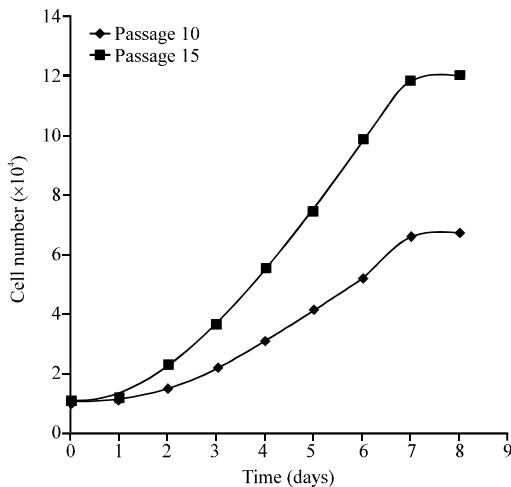

Fig. 2: Growth curves for YMM cells. YMM cells at passages 10 and cells frozen at passage 10, stored in liquid nitrogen for 3 months and then cultured for 5 more passages (passage 15) were used to study the growth characteristics. Each value represents the mean±SD of three independent experiments

phase in the following day. The population of cells grew faster from day 4-7. When extended around the plastic substratum after day 7, the number did not change obviously in the last 2 days (Fig. 2). Doubling time of YMM cells was approximately 66-68 h before passage 15.

After passage 15, YMM cells grew more quickly and their doubling time reduced to approximately 59-61 h, meanwhile their morphology changed obviously.

Chromosomal analysis of YMM cells: Chromosomal analysis of the YMM cell line supported the yak origin of these cells and showed a normal chromosomal number. Chromosomal analysis of >35 spreads of YMM cells showed a modal number of 60 (Fig. 3) which consistent with the normal diploid chromosome number for the yak species. As expected in long-term cell cultures however, chromosomal drift occurred at passage 35.

Immunocytochemical characterization of YMM cells: To further characterize the YMM cells, cells at passage 10 and 20 were examined the proteins expression of cytokeratin7, vimentin and α-smooth muscle actin. At passage 10, α-smooth muscle actin fibers were seen (Fig. 4a) in a pattern that traversed myoepithelial cells. Positive staining for vimentin (Fig. 4b) and for cytokeratin7 (Fig. 4c) were also apparent. No specific filament staining was observed for control group (Fig. 4d). At passage 20, the cells exhibited intense positive staining for α-smooth muscle actin and vimentin but weak positive staining for cytokeratin7. Cytoskeleton expression determined by immunocytochemistry was consistent with myoepithelial cells and characterized the established YMM as a homogeneous cell line.
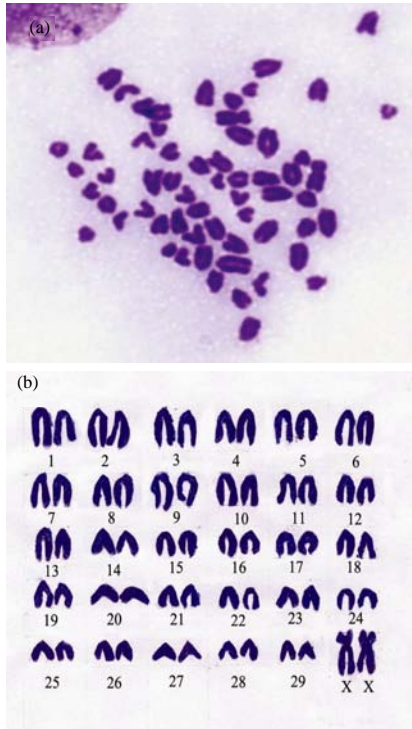
Fig. 3: Chromosome analysis of YMM cells; a) Metaphase chromosome of YMM cells (passage 25) and b) Karyotype of YMM cells (passage 25) indicates 29 pairs of telocentric chromosomes and a pair of X
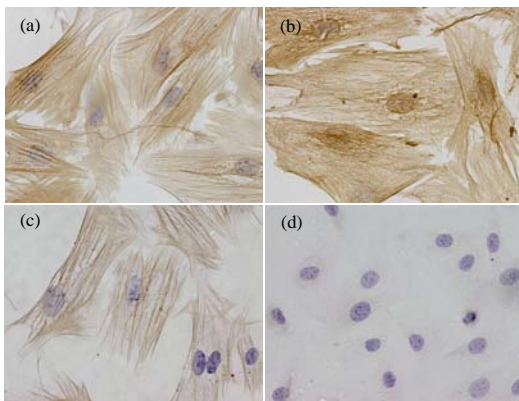

Fig. 4: Immunocytochemical analysis of YMM cells. Cells at passage 10 remain immunopositive for: a) anti-α-smooth muscle actin; b) anti-vimentin; c) anti-cytokeratin7 and d) control image identical to a-c except that the first antibody is omitted to ensure specificity of staining x400

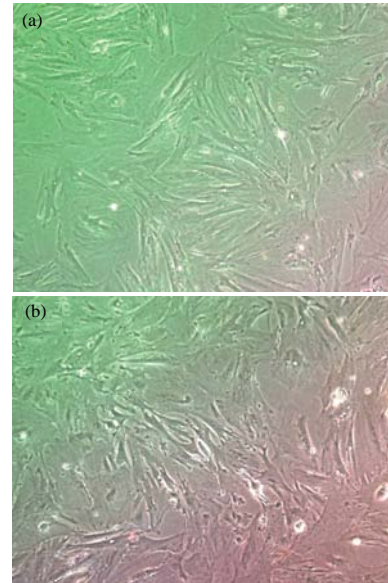**Oxytocin responsiveness:** Responsing to oxytocin of YMM cells *in vitro* were evaluated after incubation of

Fig. 5: Contraction of YMM cells in response to oxytocin. Confluent monolayers of cells; a) before and b) after addition of 50 μM of oxytocin in the culture medium x200

cells with oxytocin. Prior to the addition of oxytocin, the cells had a flattened appearance (Fig. 5a). After 30 min about 30% of the cells showed morphologic changes which mainly shown in reduction of cell size (Fig. 5b). Furthermore, some contracted cells detached from the plastic substratum and floated in suspension after 6 h incubating with oxytocin. However, the number of these cells was very little (<3%). The contractile response to oxytocin was only observed in confluent cultures. Oxytocin had no effect on any of the epithelial cell cultures nor on subconfluent YMM cell cultures.

Sterility: Cultures of YMM cells were routinely (every 10 passages) grown in the absence of penicillin and streptomycin and then evaluated for evidence of contamination by bacteria, fungus and mycoplasma. None of the cultures showed signs of contamination. The tests for bacteria and fungus contamination with THIO media were negative. YMM cells grown on coverslips were negative for contamination by Hoechst #33258 stain. The results of sheep erythrocyte agglutination test were also negative. Thus, there were no evidences of microbial contamination to the YMM cell line.

In this study, researchers described the isolation and characterization of a myoepithelial cell line from a lactational yak mammary gland. The established cell line had an advantage over primary cultures in that the cells

retained active proliferation over the relatively long period of 40 passages. Isolation of mammary cells by digestion with collagenase resulted in three types of cells with different morphology, growth capacity. During early passages we enriched the proportion of cells with myoepithelial-like morphology based on their relative easiness to detachment by trypsinization than cells with cobblestone morphology. Morphologically, YMM cells with a distinctive stellate morphology had filipodial extensions which was similar to a caprine mammary myoepithelial cell line (Pantschenko *et al.*, 2000a). However, the cells changed to polygonal-like in morphology after passage 15. The change might be due to the influence of freezing and thawing or the change of cytoskeletal protein which was evidenced by drastic reduction in the expression of cytokeratin7 using immunocytochemistry.

Myoepithelial cells exhibited expression of proteins typical for the contractile response such as α-smooth muscle actin, myosin heavy chains, α-actinin and so on (Sopel, 2010). YMM cells showed a positive staining pattern for anti α-smooth muscle actin monoclonal antibody. This result provided direct evidence that the YMM cells were myoepithelial cells as anti α-smooth muscle actin antibody specifically stained the myoepithelial cells from the bovine (Zavizion *et al.*, 1992) and caprine (Pantschenko *et al.*, 2000a) mammary gland.

Smooth muscle actin was involved in myoepithelial cells contraction and expressed in 95% of myoepithelial cells of the normal mammary gland (Bose *et al.*, 1999). Gugliotta *et al.* (1988) used anti-α smooth muscle antibody to find the optimal conditions for identification of myoepithelial cells in mammary and other several organs and they found that myoepithelial cell was the only positive epithelial cell type in these organs. The results showed that α-smooth muscle actin fibers traversed YMM cells which was in agreement with Franke *et al.* (1980a, b), Zavizion *et al.* (1992) and Pantschenko *et al.* (2000a, b).

YMM cells contained both cytokeratin and vimentin intermediate filament proteins. The presence of cytokeratin demonstrated YMM cell line as a cell of epithelial rather than mesenchymal origin. Mesenchymal cells such as fibroblast, smooth muscle (Franke *et al.*, 1978) or endothelial cells did not contain cytokeratin *in vivo* or *in vitro* (Warburton *et al.*, 1981). Vimentin fibers might function to keep the nucleus or other organelles in a definite place within the myoepithelial cell (Zavizion *et al.*, 1992). After passage 15, the expression of

cytokeratin7 was greatly reduced this might be due to YMM cells rapidly grew in media with 10% FBS. Connell and Rheinwald (1983) found rapidly growing mesothelial cells greatly reduced synthesis and content of their four major keratins to levels undetectable by immuno-fluorescence.

Vimentin was present in myoepithelial cells but not in epithelial cells of human (Rudland and Hughes, 1989) and goat (Li *et al.*, 1999) mammary gland, so vimentin was considered as a marker of myoepithelial cells *in vivo* (Ehmann *et al.*, 2003).

However, many epithelial cells in culture contained vimentin filaments (Franke *et al.*, 1979). Thus, myoepithelial cells could not be merely identified by vimentin. In the present study, we found the positive expression of three cytoskeletal proteins of α-smooth muscle actin, keratin and vimentin existed in YMM cells which were consistent with the results reported previously (Zavizion *et al.*, 1992).

Identifying myoepithelial cells with certainty was a major challenge because myoepithelial cells had a variable phenotype and could dedifferentiate (Jamieson *et al.*, 1986). Myoepithelial cells contracted in response to oxytocin which was the only hormone presently known that specifically affected the function of mammary myoepithelial cells (Sapino *et al.*, 1993). Oxytocin responsiveness was the hallmark of myoepithelial cell function (Zavizion *et al.*, 1996). We observed some YMM cells contracted after adding of oxytocin in the culture medium. This suggested that the YMM cell line was myoepithelial origin. It was not totally surprising that only about 30% of YMM cells were respond to oxytocin.

Similar observations were made in several other cell lines. Pantschenko *et al.* (2000a) found only 30-40% of the caprine mammary myoepithelial cells showed morphologic changes when maximum oxytocin responsiveness appeared. They suggested that differentiation of myoepithelial cells was associated with the non-proliferating state and implied a contact-mediated differentiation process. Zavizion *et al.* (1992) observed some cells bound oxytocin on the plasma membrane but did not contract by immunofluorescence. They thought that the affinity of the oxytocin receptors and their number likely played an important role in myoepithelial cell contraction. Several studies established that the response of myoepithelial cells to oxytocin depending on the affinity of oxytocin binding sites (Pliska *et al.*, 1986) their ultrastructure or state of differentiation (Sala and Freire, 1974) and the physiological state (Zhao and Gorewit, 1987).

CONCLUSION

This is the first study to report the establishment of a Yak Mammary Myoepithelial cell line (YMM) isolated from lactating yak mammary gland. YMM cell line may be a useful *in vitro* model for studies of the use of myoepithelial cells in mammary growth, differentiation, milk ejection and cancerogenesis of mammary gland.

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