HH2A, AN IMMORTALIZED BOVINE MAMMARY EPITHELIAL CELL LINE, EXPRESSES THE GENE ENCODING MAMMARY DERIVED GROWTH INHIBITOR (MDGI)

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SUMMARY

We have established and partially characterized a spontaneously immortalized bovine mammary epithelial cell line, designated HH2a. The cells express the gene encoding for mammary derived growth inhibitor (MDGI) when grown on released collagen gels in the presence of lactogenic hormones. This is the first report of a cell line that expresses MDGI. Immunohistochemical studies showed that HH2a cells contain keratin intermediate filaments and desmosomes. When plated on confluent monolayer of live fibroblasts, HH2a cells extensively contacted with fibroblasts. When embedded in the collagen gels, they rearranged themselves to produce three-dimensional duct-like outgrowths extending into the matrix. The HH2a cell line should be useful in investigations of the roles of cell-cell and cell-extracellular interactions in regulation of breast epithelial cell proliferation, and of the hormonal regulation of MDGI gene expression.

Key words: immortalized; mammary epithelial cells; MDGI.

INTRODUCTION

In vivo, the mammary epithelium exists within a complex stromal environment. Along with the mammary stromal fibroblasts are a number of other cell types that have potential roles in normal mammary gland function. The organization and function of mammary epithelium are markedly influenced by substrata on which these cells are cultured. It has been demonstrated that collagen gels (13,20), mammary gland biomatrix (32), and basal lamina components extracted from Engelbreth-Holm-Swarm tumors (21) can induce mammary gland differentiation.

Upon pregnancy and lactation, the ductal cells differentiate into alveolar cells capable of milk secretion. During the pathogenesis of breast cancer, normal epithelial cells can give rise to morphologically altered alveolar and ductal dysplasias. Both alveolar dysplasias, termed "preneoplastic hyperplastic alveolar nodules", and the ductal dysplasias, in turn, have a high probability of progressing to neoplastic state (23). Considerable effort has been directed toward the development of cell culture methodology for establishing cell lines of mammary origin. To date, only a few mammary cell lines of epithelial origin have been established (1,2,11,14,17,18,28,31). Of these, only the COMMA-1D line could be induced to synthesize an appreciable amount of casein in vitro (11). Subcloning of COMMA-1D gave rise to several cell types (2,10,24). Prolonged passage of COMMA-1D cells led to increased oncogenic potential (24).

MDGI has been partially purified from mammary tissue of a lactating cow (6). It has been demonstrated that partially purified bovine MDGI inhibited human mammary ascites cells (5), and human Matu breast cancer cells at a concentration of 10^{-9} *M* (5). Antisera against MDGI were raised (7) and the complete peptide sequence was reported (4). Immunolocalization using specific antisera against bovine MGDI revealed that MDGI was localized not only in the cytosolic fraction but also in nuclear fraction (26). MDGI was also detected in milk (8). In 1990, cDNA for bovine MDGI was cloned from a plasmid library derived from mammary tissue of a lactating cow (19) and mRNA was localized in terminally differentiated mammary epithelial cells (26). Recently, hormonal regulation of mouse MDGI by prolactin, insulin, and cortisone has been demonstrated in vitro using mouse mammary organ culture (3).

To date, no mammary epithelial cell lines have been reported to express mammary derived growth inhibitor in vitro. In this report, we partially characterize an immortalized bovine mammary epithelial cell line that exhibits several properties of normal mammary epithelial cells including expression of MDGI gene.

MATERIALS AND METHODS

Cells. Primary bovine mammary gland cells were obtained by aseptic biopsy from a pregnant Holstein heifer at slaughter and dissociated according to the procedure of Burwen and Pitelka (9). Mammary tissue was first washed three times with cold Dulbecco's phosphate buffer saline (DPBS, GIBCO, Grand Island, NY). It was minced with a shearing motion with scalpel blades under aseptic conditions. The minced tissue was suspended 1:10 (wt/vol) dissociation medium [1× Hanks' buffer saline solution (GIBCO), 11 m*M* glucose, 4% serum albumin (Sigma, St. Louis, MO), and 200 IU/ml collagenase (Sigma)] in a trypsinizing flask. The flask was rotated at 200 rpm for 60 min in the water bath at 37° C. The separation of the cells from connective tissue was further enhanced by the mechanical disruption by repeating pipetting. Single cells and clusters of cells were obtained by repeated filtration through 150 μm Nitex cloth (Technico, Montreal, PQ). Cells and cell clumps were collected by centrifugation at 800 ×*g* for 2 min, then washed by resuspension in DPBS.

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Cells were seeded at the density of 5×10^4 cells per cm^2 on plastic petridish in the Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal calf serum (FCS, GIBCO), 5 μg/ml insulin (Sigma), 1 μg/ml hydrocortisone (Sigma). Cells were incubated at 37° C in a 5% CO_2 and 100% humidity atmosphere. Overgrowing fibroblasts were removed each week by differential trypsinization. The growth of epithelial colonies were apparent after 24 wk of culture. Cells were split 1 to 5 at weekly intervals. After surviving growth crisis, colonies were selected and subjected to a second cycle of cloning by limiting dilution, after which the cytoskeleton of each of the clones was analyzed. Selected colonies were tested for mycoplasma contamination. All colonies were mycoplasma free.

To study stromal-epithelial interaction, primary bovine fibroblasts at the Passage 6 were used for co-culture experiments. Cells were plated 4 d prior to the addition of epithelial cells. HH2a cells were seeded at the density 10×10^6 cells per 100 mm plate in differentiation media [DMEM containing 5% FCS (GIBCO), 5 μg/ml insulin (Sigma), 1 μg/ml hydrocortisone (Sigma), and 1 μg/ml bovine prolactin (USDA-6 PRL B-I)]. Media was changed daily for 5 d. Structural organization of the co-culture was recorded daily.

To study the effect of cell-extracellular interaction, HH2a cells were plated on monolayer collagen gels that were then overlaid with another layer of collagen. The collagen gels were released to float 24 h later. Cells were allowed to grow in differentiated media for 5 d. Structural organization was recorded daily.

To test the ability of HH2a cells to express MDGI mRNA, HH2a cells were grown either on plastic or collagen gels in the presence of prolactin. Calf tail collagen was prepared according to the methods of Michalopoulos and Pitot (25). The collagen gels were sterilized under UV light for 2 h and equilibrated for 1 h in phosphate buffer saline (PBS) followed by an overnight in DMEM (GIBCO) supplemented 5 μg/ml insulin (Sigma), 10% FCS (GIBCO). HH2a cells at Passage 72 were used in these experiments. Cells were plated either on plastic culture petri dish or on calf tail collagen gels at a density of 5×10^5 cells/cm^2. After 24 h, media was changed and appropriate media was replaced. For control plates, cells were grown in basal media (DMEM containing 5% FCS, 5 μg/ml insulin, 1 μg/ml hydrocortisone). For differentiation, cells were grown in the differentiation media described above. Collagen gels were left intact or released to float. For RNA extraction, collagen gels containing cells were harvested for 4 d after collagen gels were released.

RNA isolation and Northern blot analysis for MDGI mRNA. Total RNA was extracted using RNAzol premixed solution and RNAzol B (Tel-test, Friendswood, TX) method. Twenty micrograms of total RNA were electrophoresed on a 1.2% agarose gel containing 2.2% formaldehyde in 1× MOPS (22) buffer for 3 h at 60 volts (22). After electrophoresis, the gels were stained with ethidium bromide and photographed under UV illumination. Gels were capillary blotted overnight onto nylon membranes (Zeta-probe, Bio-Rad, Richmond, CA) in 50 m*M* NaOH. Filters were prehybridized overnight in the solution, consisting of 50% deionized formamide, 150 mg/ml dry skim milk powder, 5× SSPE (22), 1% SDS, 500 μg/ml denatured herring sperm DNA (Boehringer Mannheim, Montreal, PQ). Hybridization was carried out in the above solution containing 10% dextran sulphate and the denatured ^{32}P labeled bovine MDGI cDNA insert (19) or bovine β-actin cDNA insert (12). Probes were randomly labeled with ^{32}P (ICN, Irvine, CA) using oligolabeling kit (Pharmacia, Montreal, PQ). Unincorporated ^{32}P dCTP was removed using a Nick-Column (Pharmacia) according to the suppliers' instructions. Specific activity was typically $1-2 \times 10^8$ dpm/μg DNA. Post-hybridization washes consisted of 15 min washes in 2× SSC (22), then 0.5× SSC and 0.1× SSC each one containing 0.1% SDS at 42° C. The final wash was carried out in 0.1 × SSC, 0.1% SDS at 60° C for 30 min. Filters were air-dried and exposed to X-Omat film between Cronex Lighting-plus intensifying screens (Dupont, Montreal, PQ) at −70° C for 1 to 5 d.

Immunohistochemistry. Cells were grown in an 8-chamber slide (Lab-Tex 4838–8 chamber Fixed Gasket, GIBCO) at the density of 1×10^3 cells per chamber. Twenty-four or seventy-two hours later, cells were fixed in cold acetone at −20° C for 10 min, then air dried to remove excess acetone. Cells were stained with either mouse anti-cytokeratin no. 1–8 (Boehringer Mannheim) or mouse anti-vimentin (Boehringer Mannheim), according to the supplier's instructions for 60 min at 37° C in a 100% humidity chamber. At the end of the incubation, each slide was washed three times in DPBS for 5 min each at room temperature. Immunofluorescent localization of the primary antibody was accomplished using a sheep anti-mouse-IgG conjugated to FITC (Boehringer Mannheim). The incubation conditions and

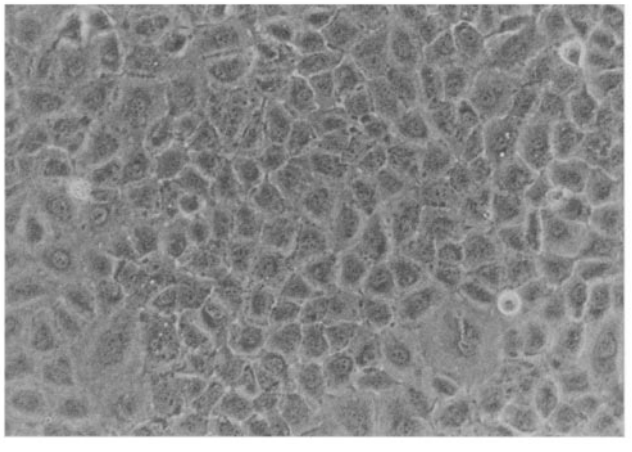

FIG. 1. Monolayer of HH2a cells at saturation density (phase contrast; ×200).

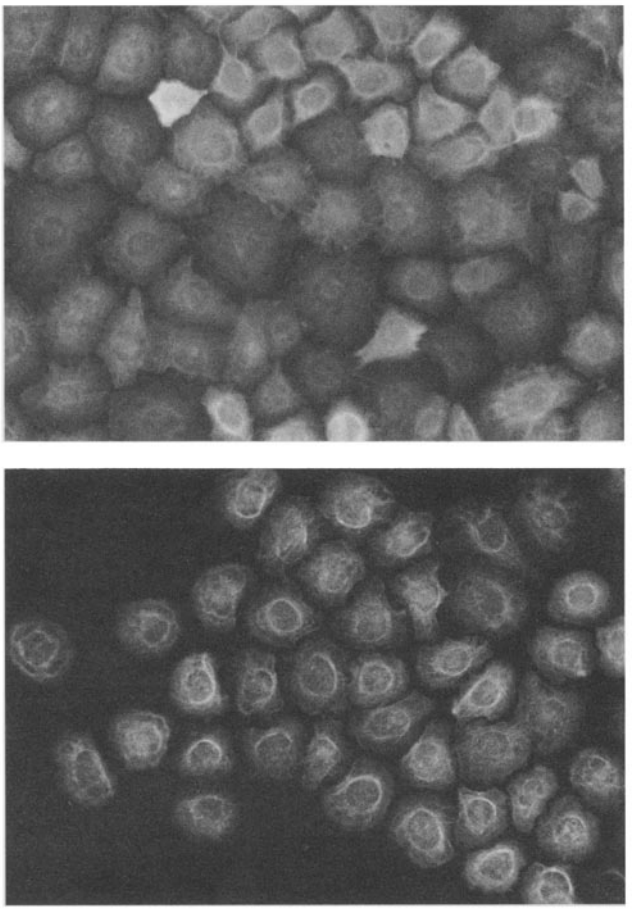

FIG. 2. Immunofluorescent image cytokeratin in HH2a cells. *A*, At high density, HH2a cells showed fibril patterns between adjacent cells, associated with desmosome containing bridges. *B*, At low density, HH2a cells showed a dense meshwork of cytokeratin fibrils. These characteristics are unique to epithelial cells (Phase contrast; ×200).

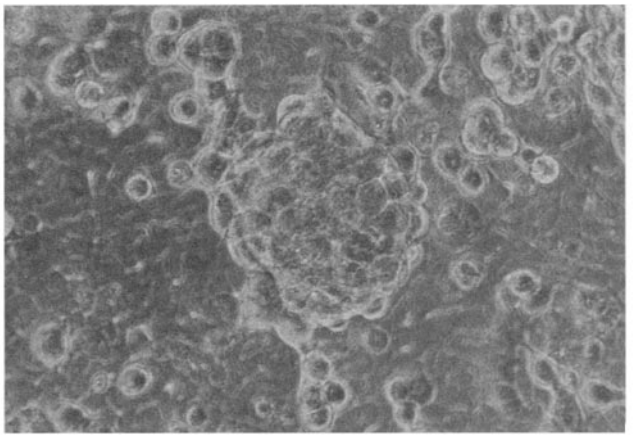

FIG. 3. Growth of HH2a cells on released collagen gels. *Note* the dome structure and columnar shape of HH2a cells within these structures (phase contrast; ×200).

washing were performed as above. Slides were visualized with a Jenalunar microscope equipped with epifluorescent optics for FITC.

RESULTS AND DISCUSSION

HH2a cells retained the phenotypic characteristics of a bovine epithelial cell line, even after multiple subcultures in vitro. On plastic, HH2a cells displayed typical cuboidal epithelial morphology in culture and formed a monolayer with other cells in close contact, tightly adhering to the plastic substratum (Fig. 1). Typical display of a major cytoskeletal component of HH2a cells was illustrated by immunofluorescent microscopy in Fig. 2. HH2a cells showed intense staining of the cytoplasmic meshwork of cytokeratin fibrils. Cells at confluency showed typical interbridges that contained desmosomes (Fig. 2 *A*). When HH2a cells were grown at low density, they did not exhibit spatial foci of association of cytokeratin bundles with the cell periphery, but rather showed a dense uniform cytoplasmic meshwork of cytokeratin fibrils (Fig. 2 *B*). HH2a cells did not stain with anti-vimentin (data not shown). These results are consistent with previous reports for bovine mammary epithelial cell lines (29). Positive immunostaining for cytokeratin and absence of vimentin suggested that HH2a cells were epithelial.

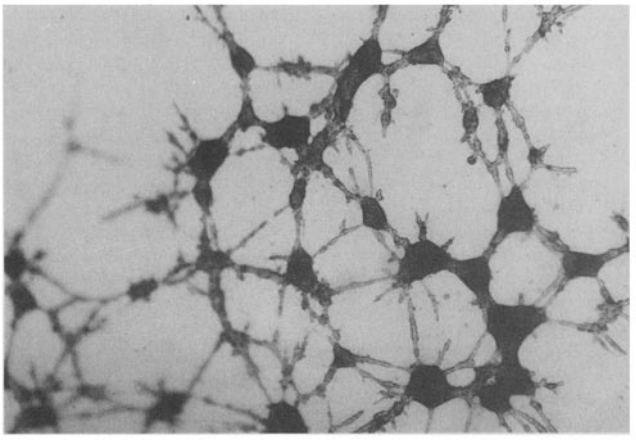

FIG. 4. Outgrowth of HH2a cells embedded in a collagen gel (PHase contrast; ×100). *Note* the duct-like morphology of the outgrowth and spherical clusters.

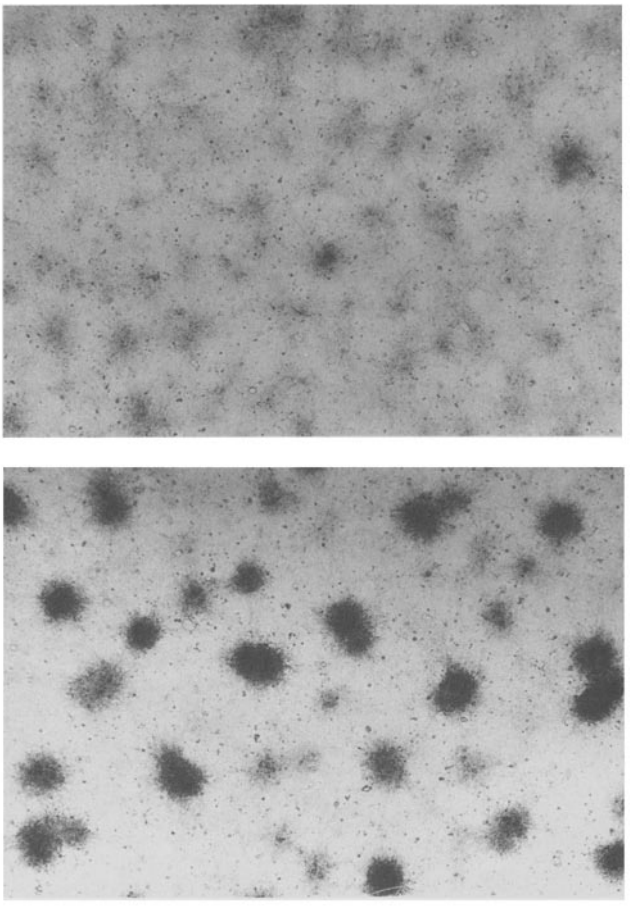

FIG. 5. Growth characteristics of HH2a cells on confluent monolayers of live fibroblasts. *A*, Day 1 after plating. HH2a colonies display stellate contours and fibroblasts are seen in contact with HH2a but are randomly oriented (phase contrast ×60). *B*, Day 5 of culture, fibroblasts showed extensive contact with HH2a cells (phase contrast ×60).

When HH2a cells were grown on collagen gels, they rearranged themselves to dome structures (Fig. 3). Cells within these structures became more columnar in shape. These characteristic arrangements resemble the in vivo organization of the bovine mammary alveolus. Dome formation has been previously reported in the murine mammary epithelial cell line, COMMA-1D (11) and was thought to be due to unidirectional fluid secretion of polarized epithelial cells when cultured on a plastic (30). These morphological characteristics suggest that HH2a cells may originate from luminal epithelial cells of the mammary gland alveolus.

When HH2a cells embedded in the collagen gels, they formed three-dimensional arrangements, including branching tubules, and cells rearranged themselves to produce three-dimensional duct-like outgrowths extending into the matrix (Fig. 4). These cells also formed circular or spherical clusters surrounding a small lumina (Fig. 4), which appeared morphologically similar to acini of glandular tissue. Similar observations have been reported for mouse mammary epithelial cells (15,33,34).

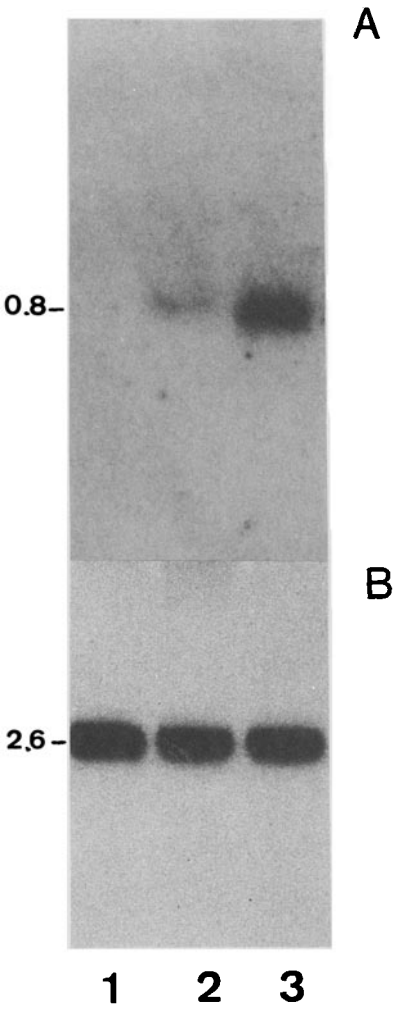

FIG. 6. Northern blot analysis of bovine MDGI mRNA. Total RNA (30 μg/lane) obtained from HH2a cells grown on plastic (*lane 1*), on nonreleased collagen gels (*lane 2*), and on released collagen gels (*lane 3*) in the presence of insulin, cortisone, and bovine prolactin. Blots were hybridized with ^{32}P-labeled bovine MDGI cDNA insert (*A*) or bovine β-actin cDNA insert (*B*).

When HH2a cells were plated on a confluent monolayer of live fibroblasts, little direct contact of the two cell types was observed on the first days (Fig. 5 *A*). After 5 d of culture, the cells were extensively in contact with each other and clusters of epithelial cells developed on the top of the fibroblastic monolayer (Fig. 5 *B*), suggesting that interaction between the two cell types is required for the formation of this characteristic structure. A requirement of fibroblasts and epithelial cell interaction in promotion of estrogen-dependent DNA synthesis (16) and β-casein gene expression has been reported (27).

To quantify the amount of MDGI mRNA in differentiated HH2a cells, denatured total RNA was run in a 1.2% gel, transferred onto Zeta-probe membrane, and hybridized with ^{32}P labeled bovine MDGI cDNA (19). The patterns of MDGI and β-actin mRNA in mammary epithelial cells grown on plastic, intact collagen and floating collagen gels in the presence of prolactin are shown in Fig. 6. MDGI mRNA was undetectable when cells were grown on plastic substratum, and were detectable only at a very low level on nonreleased collagen gels (Fig. 6 *A*). However, the MDGI transcripts were readily detected after collagen gels were released to float (Fig. 6 *A*). Subsequently, hybridization on the same blot with bovine β-actin probe showed that relatively equal amounts of intact RNA were present in each lane (Fig. 6 *B*). The results suggested that HH2a cells are still responsive to extracellular matrix in the presence of lactogenic hormones even though they were extensively passaged in vitro. Attempts to detect MDGI mRNA in several human breast cancer cell lines such as MCF-7, T47D, MDA 231, BT20, as well as the non-transformed bovine MAC-T mammary epithelial cell line showed an absence of MDGI transcripts (data not shown). The ability of HH2a cells to express MDGI appears to be unique for this cell line.

HH2a provides an example of a non-transformed immortalized breast epithelial cell line. This cell line has been growing for more than 1 yr through more than 160 passages. The cells grow in a stable fashion and show no signs of senescence. Even after multiple subcultures in vitro, HH2a cells retain some differentiated characteristics of normal mammary epithelial cells, including the ability to respond to lactogenic hormones and extracellular matrix, and to express mRNA encoding MDGI. The availability of this established bovine mammary epithelial cell line should prove useful for studying cell-cell and cell-extracellular matrix interactions, as well as hormonal regulation and physiological consequences of MDGI expression in vitro.

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REFERENCES

1. Anderson, L. W.; Danielson, K. D.; Hosick, H. Epithelial cell line and subline established from premalignant mouse mammary tissue. In Vitro Cell. Dev. Biol. 15:841–843; 1979.
2. Ball, R. K.; Friis, R. R.; Schoenenberger, C. A., et al. Prolactin regulation of β-casein gene expression and of a cytosolic 120 kDa protein in a cloned mouse mammary epithelial cell line. EMBO J. 7:2089–2095; 1988.
3. Binas, B.; Spitzer, E.; Zschiesche, W., et al. Hormonal induction of functional differentiation and mammary derived growth inhibitor expression in cultured mouse mammary gland explants. In Vitro Cell. Dev. Biol. 28A:625–634; 1992.
4. Bohmer, F. D.; Kraft, R.; Otto, A., et al. Identification of a polypeptide growth inhibitor from bovine mammary gland. J. Biol. Chem. 262:15137–15143; 1987.
5. Bohmer, F. D.; Lehmann, W.; Noll, F., et al. Specific neutralizing antiserum against a polypeptide growth inhibitor for mammary cells purified from bovine mammary gland. Biochim. Biophys. Acta. 846:145–154; 1985.
6. Bohmer, F.; Lehman, W.; Schmidt, H. E.; et al. Purification of a growth inhbitor for ehrlich ascites mammary carcinoma cells from bovine mammary gland. Exp. Cell. Res. 150:466–476; 1984.
7. Bohmer, F. D.; Sun, Q.; Pepperle, M., et al. Antibodies against mammary derived growth inhibitor (MDGI) react with a fibroblast growth inhibitor and with heart fatty acid binding protein. Biochem. Biophys. Res. Commun. 148:1425–1431; 1987.
8. Brandt, R.; Pepperle, M.; Otto, A., et al. A 13-kilodalton protein purified from milk fat globule membranes is closely related to a

mammary-derived growth inhibitor. Biochem. 27:1420–1425; 1988.
9. Burwen, S. J.; Pitelka, D. Secretory function of lactating mouse mammary epithelial cells culture on collagen gels. Exp. Cell. Res. 126:249–262; 1980.
10. Campbell, S. M.; Taka, M. M.; Medina, D., et al. A clonal derivative of mammary epithelial cell line COMA-D retains stem cell characteristics of unique morphological and functional heterogeneity. Exp. Cell. Res. 177:109–121; 1988.
11. Danielson, K. G.; Oborn, C. J.; Durban, E. M., et al. Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. Proc. Natl. Acad. Sci. USA 81:3756–3760; 1984.
12. Degen, J. L.; Neubauer, M. G.; Degen, S. J. F., et al. Regulation of protein synthesis in mitogen-activated bovine lymphocytes. J. Biol. Chem. 258:12153–12162; 1983.
13. Emerman, J. T.; Pitelka, D. R. Maintenance and induction of morphological differentiation in dissociated mammary epithelia on floating collagen membranes. In Vitro 13:316–328; 1977.
14. Ghosh, S. K.; Roholt, O. A.; Kim, U. Establishment of two non-metastasizing and one metastasizing rat mammary carcinoma cell lines. In Vitro Cell. Dev. Biol. 19:919–927; 1993.
15. Hall, H. G.; Farson, D. A.; Bissell, M. J. Lumen formation by epithelial cell lines in response to collagen overlay: a morphogenetic model in culture. Proc. Natl. Acad. Sci. USA 79:4672–4676; 1982.
16. Haslam, S. Z. Cell to cell interaction and normal mammary gland function. J. Dairy Sci. 108:1127–1138; 1988.
17. Howard, D. K.; Schlom, J.; Fisher, P. B. Chemical carcinogen-mouse mammary tumor virus interactions in cell transformation. In Vitro Cell. Dev. Biol. 19:58–65; 1983.
18. Huynh, T. H.; Robitaille, G.; Turner, J. D. Established of bovine mammary epithelial cell line (MAC-T): an in vitro model for bovine lactation. Exp. Cell. Res. 197:191–199; 1991.
19. Kurtz, A.; Vogel, F.; Funa, K., et al. Developmental regulation of mammary-derived growth inhibitor expression in bovine mammary tissue. J. Cell. Biol. 110:1779–1789; 1990.
20. Lee, E. Y. H.; Lee, W.; Keatzel, C. S., et al. Interaction of mouse epithelial cells with collagen substrata: regulation of casein gene expression. Proc. Natl. Acad. Sci. USA 82:1419–1423; 1985.
21. Li, M. L.; Aggeler, J.; Farson, D. A., et al. Influence of a reconstituted basement membrane and its components in casein gene expression and secretion in mouse mammary epithelial cells. Proc. Natl. Acad. Sci. USA 84:136–140; 1987.
22. Maniatis, T.; Fritsch, E. F.; Shambrook, J. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1982.
23. Medina, D. Breast cancer. Vol. 2. New York: Plenum; 1978:47–102.
24. Medina, D.; Oborn, C. J.; Kittrell, F. S., et al. Properties mouse mammary epithelial cell lines characterized by in vivo transplantation and in vitro immunocytochemical methods. JNCI 76:1143–1151; 1986.
25. Michalopoulos, G.; Pitot, H. C. Primary culture of parenchymal liver cells on collagen membranes. Exp. Cell. Res. 94:70–78; 1975.
26. Muller, T.; Kurtz, A.; Vogel, F., et al. A mammary-derived growth inhibitor (MDGI) related 70 kDa antigen identified in nuclei of mammary epithelial cells. J. Cell. Physiol. 138:415–423; 1989.
27. Reichmann, E.; Ball, R.; Groner, B., et al. New mammary epithelial and fibroblastic cell clones in coculture form structures competent to differentiate functionally. J. Cell. Biol. 108:1127–1138; 1989.
28. Schmid, E.; Franke, W. W.; Grund, C., et al. An epithelial cell line with elongated myoid morphology derived from bovine mammary gland. Exp. Cell. Res. 146:309–328; 1983.
29. Schmid, E.; Schiller, D. L.; Grund, C., et al. Tissue type-specific expression of intermediate filament proteins in a cultured epithelial cell line from bovine mammary gland. J. Cell. Biol. 96:37–50; 1983.
30. Sugahara, K.; Caldwell, J. H.; Mason, R. Electrical currents flow out of domes formed by cultured epithelial cells. J. Cell. Biol. 99:1541–1546; 1984.
31. Van Deurs, B.; Zou, Z. Z.; Briand, P., et al. Epithelial membrane polarity: a stable, differentiated feature of an established human breast carcinoma cell line MCF-7. J. Histochem. 35:461–469; 1987.
32. Wicha, M. S.; Lowrie, G.; Kohn, E., et al. Extracellular matrix promotes mammary epithelial growth and differentiation in vitro. Proc. Natl. Acad. Sci. USA 79:3213–3217; 1982.
33. Yang, J.; Richards, J.; Bowman, P., et al. Sustained growth and three-dimensional organization of primary mammary tumour epithelial cells embedded in collagen gels. Proc. Natl. Acad. Sci. USA 76:3401–3405; 1979.
34. Yang, J.; Richards, J.; Guzman, R., et al. Sustained growth in primary culture of normal mammary epithelial cells embedded in collagen gels. Proc. Natl. Acad. Sci. USA 77:2088–2092; 1980.