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13. ABSTRACT (Maximum 200 Words)
In glandular organs, a precancerous state precedes invasive carcinoma. In the breast, this state is recognized as DCIS and consist of an epithelial cell proliferation confined by myoepithelial cells. Our laboratory has established cell lines/xenografts of myoepithelial cells. Our myoepithelial cell lines inhibit invasion and motility of breast carcinoma line *in vitro* largely through maspin. The overall hypothesis of this proposal was how does myoepithelial maspin regulate breast (DCIS) carcinoma progression and can its detection in fine needle aspirates (FNA) and in ductal fluid abet diagnosis and screening? The first aim addressed the mechanism of maspin's inhibition of breast carcinoma invasion: We have shown that myoepithelial maspin does bind to plasma membranes of carcinoma cells and inhibit a pathway involved in cellular locomotion. Using mRNA expression profiling we have further characterized the pathway(s) involved. Furthermore we have shown that myoepithelial cells exhibit by mRNA expression profiling a common tumor-suppressor phenotype. The second aim utilized maspin antibodies on FNA to identify the myoepithelial component and we have further demonstrated prospectively with larger number of samples that this approach is successful. The third aim investigated the levels of maspin in nipple aspirates, in ductal lavage fluid and saliva. We have shown that maspin is both a tumor marker as well as a surrogate intermediate end point marker.

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INTRODUCTION

In many glandular organs, a precancerous state is thought to exist which precedes the development of frankly invasive carcinoma. In the breast this state is recognized as DCIS and consists of a proliferation of epithelial luminal cells confined by myoepithelial cells within the ductal system (1-7). CGH and LOH studies have failed to demonstrate a difference between DCIS and invasive breast carcinoma. Our laboratory has recently demonstrated that paracrine regulation of this transition by myoepithelial cells may be the main determinant of this important step in human breast carcinoma progression. Because of their close proximity to precancerous lesions, myoepithelial cells would be expected to exert important paracrine influences on these processes. Myoepithelial cells of the breast differ from ductal cells in many ways: they lack ER-α, and its downstream genes; they synthesize the adjacent basement membrane; they rarely proliferate or fully transform and give rise rarely only to low grade benign neoplasms. Myoepithelial cells are present around normal ducts and precancerous proliferations but are absent in invasive carcinoma. Our laboratory has established immortalized myoepithelial cell lines and xenografts from benign human myoepitheliomas of the salivary gland (HMS-1, HMS-3) and breast (HMS-4,5) (8-23). These cell lines and xenografts express identical myoepithelial markers as their *in situ* counterparts. Our myoepithelial cell lines and xenografts and myoepithelial cells *in situ* constitutively express high amounts of proteinase and angiogenesis inhibitors which include TIMP-1, protease nexin-II, α-1 antitrypsin, an unidentified perhaps novel 31-33 kDa trypsin inhibitor (24), thrombospondin-1, soluble bFGF receptors, and maspin (25-34). The human myoepithelial cell lines, HMS-1, HMS-3, HMS-4, HMS-5 inhibit both ER-positive and ER-negative breast carcinoma invasion (down to 42%$\pm$7% of control) ($p<.05$) and in CM assays (down to 30%$\pm$8% of control) ($p<.01$).

The anti-invasive effects of HMS-1, HMS-3, HMS-4, HMS-5 and HMS-6 can be enhanced by phorbol 12-myristate 13-acetate (PMA) (down to 2%$\pm$1% of control) and abolished by dexamethasone (up to 95%$\pm$5% of control) ($p<.01$) Therefore with the appropriate pharmacological treatment, the myoepithelial cells do not only partially inhibit invasion --- they in fact near-totally eliminate it. PMA treatment causes an immediate and sustained release of maspin, a recently identified serpin. Immunoprecipitation of maspin from this CM nearly abolishes this anti-invasive effect. Adding purified native myoepithelial maspin to unconditioned media also results in a dramatic anti-invasive effect. Maspin exerts a similar inhibitory effect on breast carcinoma cell motility as noted by us using native maspin and others using both recombinant bacterial maspin and recombinant insect maspin(i) produced in Baculovirus-infected insect cells (35). Our findings suggest that myoepithelial-secreted maspin functions as a paracrine tumor suppressor, which may inhibit *in vivo* the progression of DCIS to invasive breast carcinoma.

The observation that myoepithelial cells express and secrete maspin has, in addition to these biological implications, important potential practical applications. Fine needle aspiration cytology of the breast is a safe noninvasive technique for diagnosing breast cancer that is being used with increasing frequency in older women and women with comorbid disease who would benefit from being spared a more invasive and anesthesia-requiring procedure such as lumpectomy. Presently however there is no way on FNA of distinguishing invasive breast cancer cells from DCIS cells. This is because both types of cells appear cytologically identical and on routine FNA there is no way to evaluate the cells in the anatomical context of the tissues. We feel

however that if myoepithelial cells could be selectively identified on FNA then their presence would suggest the DCIS state since they would be expected to be aspirated along with the DCIS epithelial cells. Their absence on the smear would suggest that the malignant cells which were present were derived from invasive carcinoma cells which are devoid of surrounding myoepithelial cells *in vivo*. Perhaps the absolute number or density of myoepithelial cells would also be discriminating factors. Our laboratory has shown that the immunocytochemical demonstration of maspin reliably distinguishes myoepithelial cells from all epithelial cells (normal, DCIS, and invasive carcinoma). Furthermore our laboratory has detected maspin in ductal fluid of the breast obtained by both nipple suction and selective breast ductal cannulation (36). This maspin in ductal fluid is produced by myoepithelial cells *in vivo* and reflects the integrity of the normal ductal lobular unit. Conceivably reduced levels of maspin in ductal fluid may reflect either structural or functional compromise of the myoepithelial layer and may be seen in high risk *v* normal women and/or ducts with abnormal microcalcifications, precancerous or invasive histopathology (37-40).

BODY (STATEMENT OF WORK)

1. To investigate the mechanism of maspin's inhibition of breast carcinoma cell invasion and motility. - Months 1-36

A. Maspin binding to breast carcinoma cells/plasma membranes. - Months 1-6

B. Identification and characterization of a maspin binding protein.- Months 6-18

C. Maspin activation of a breast carcinoma cellular pathway.- Months 18-36

We have continued our previous observations made during the second year into the third year of funding that purified myoepithelial maspin inactivates a cellular pathway involved in cellular locomotion. In addition we have utilized mRNA expression profiling of our myoepithelial tumors to demonstrate potential myoepithelial cellular pathways involved in maspin secretion and tumor suppression.

2. To utilize antibodies to maspin on fine needle aspirate (FNA) specimens to quantitate the myoepithelial component and determine if this determination discriminates between DCIS and invasive breast carcinoma. - Months 12-24

In year 02, we extended our previous observations by expanding them to a much larger number of cases and further demonstrated that antibodies to maspin (polyclonal and monoclonal) are the best discriminator between myoepithelial cells and epithelial cells and they can be used to quantitate the myoepithelial component in FNA. In year 03 we began a prospective study using this same approach. We plan to continue this prospective approach in a future grant application.

3. To investigate the levels of maspin in ductal fluid obtained by the nipple suction approach (in high risk *v* normal women) and in ductal fluid obtained by selective ductal cannulation and washings (ducts with and without microcalcifications from the same

patient) and determine whether maspin levels abet screening and correlate with histological findings/ - Months 24-36

We had previously demonstrated that nipple aspirate fluids and ductal lavage fluids obtained by ductoscopy is rich in maspin as a surrogate intermediate end point marker that reflected the integrity of the ductal lobular unit. The methods used in these studies were contained in our publications (41,42). Ductal fluid contains numerous proteins in addition to maspin such as bFGF which may also be a marker for early cancerous changes. These points were made in another of our publications (43). Access to the ductal system of the breast and specifically targeting the myoepithelial-epithelial connection through gene therapy intraductal approaches may prove efficacious in early breast cancer chemoprevention and treatment, strategies initially claimed in our patent application (44) which has now become an official U.S. patent (45). In year 03 we focused more closely on this aim, expanding the number of cases and correlating the levels of maspin with the structural integrity of the ductal lobular unit. Our findings indicated that when the integrity of the ductal lobular unit is preserved the levels of maspin are not influenced by the degree of epithelial proliferation. The levels of other markers, eg. CEA, PSA, bFGF are however. When there is invasive cancer however and the myoepithelial layer is transgressed and / or destroyed, the level of maspin decreases or is absent. We plan to continue these observations in a prospective randomized trial which will be the subject of a subsequent grant proposal.

KEY RESEARCH ACCOMPLISHMENTS

- We have further demonstrated during the third year of funding that purified myoepithelial maspin binds to plasma membranes of carcinoma cells via a membrane receptor and both inactivates a cellular pathway involved in cellular locomotion and activates several other pathways determined by mRNA expression profiling. We are further characterizing these pathways presently. A series of myoepithelial cell lines and xenografts derived from benign human myoepithelial tumors of diverse sources (salivary gland, breast and lung) exhibit common mRNA expression profiles indicative of a tumor suppressor phenotype. Previously established myoepithelial cell lines and xenografts (HMS-#; HMS-#X) were compared to non-myoepithelial breast carcinoma cells (MDA-MB-231, MDA-MB-468 and inflammatory breast carcinoma samples: IBCr, IBCw), a normal mammary epithelial cell line (HMEC) and individual cases of human breast cancer (zcBT#T) and matched normal human breast tissues (zcBT#N) (overall samples = 22). The global gene expression profile (22,000 genes) of these individual samples were examined using Affymetrix Microarray Gene Chips and subsequently analyzed with both Affymetrix and DChip algorithms. The myoepithelial cell lines / xenografts were distinct and very different from the non-myoepithelial breast carcinoma cells and the normal breast and breast tumor biopsies. 207 specifically selected genes represented a subset of genes that distinguished ($p<0.05$) all the myoepithelial cell lines / xenografts from all the other samples and which themselves exhibited hierarchical clustering. Further analysis of these genes revealed increased expression in genes belonging to the classes of extracellular matrix proteins, angiogenic inhibitors and proteinase inhibitors and decreased expression belonging to the classes of angiogenic factors and proteinases. Developmental genes were also differentially expressed (either over or underexpressed).

These studies confirm our previous impression that human myoepithelial cells express a distinct tumor suppressor phenotype.

- We have further demonstrated using larger numbers of samples that antibodies to maspin (polyclonal and monoclonal) are the best discriminator between myoepithelial cells and epithelial cells and they can be used to quantitate the myoepithelial component. This can discriminate between pure DCIS and DCIS associated with invasion. Prospective studies testing this discrimination have begun in year 03 and will be the focus of a subsequent grant proposal.

- We have demonstrated that nipple aspirate fluids and ductal lavage fluids obtained by ductoscopy is rich in maspin as a surrogate intermediate end point marker that reflects the integrity of the ductal lobular unit. Ductal fluid contains numerous proteins in addition to maspin such as bFGF which may also be a marker for early cancerous changes. Access to the ductal system of the breast and specifically targeting the myoepithelial-epithelial connection through gene therapy intraductal approaches may prove efficacious in early breast cancer chemoprevention and treatment, strategies claimed in our patent application. We have further confirmed these points by studying more samples in year 03 of the study. Our findings indicated that when the integrity of the ductal lobular unit is preserved the levels of maspin are not influenced by the degree of epithelial proliferation. The levels of other markers, eg. CEA, PSA, bFGF are however. When there is invasive cancer however and the myoepithelial layer is transgressed and / or destroyed, the level of maspin decreases or is absent. We plan to continue these observations in a prospective randomized trial which will be the subject of a subsequent grant proposal.

REPORTABLE OUTCOMES

PUBLICATIONS

1. **Barsky SH** and Alpaugh ML. Myoepithelium: Methods of culture and study. Culture of Human Tumor Cells, edited R. Ian Freshney, John Wiley & Sons, Inc., New York, NY, 2003

2. **Barsky SH.** Myoepithelial mRNA expression profiling reveals a common tumor suppressor phenotype. *Experimental and Molecular Pathology* 74: 113-122, 2003.

PATENTS AND LICENSES

1. **Barsky SH** and Alpaugh ML. Compositions and methods for intraductal gene therapy. *U.S. Patent* 6,514,695; February 4, 2003

CONCLUSIONS

1. Mechanism of Maspin Action

Maspin activation / inactivation of a cellular pathway. The first question that we addressed was whether maspin exhibits specific, saturable, reversible, and displaceable binding to the surface of breast carcinoma cells in a manner of a ligand-receptor interaction and it does. The mechanism of maspin's effects on invasion and motility inhibition are still unknown. Our studies have shown that in myoepithelial cells it is *secreted* in large amounts. We have been able to purify native maspin to homogeneity. We have obtained *r*maspin from Dr. Zhang (Baylor). Both *r*maspins (bacterial and insect) and native maspin derived from myoepithelial cells have been iodinated and incubated with first intact MDA-231, MDA-468, MCF-7, T47D cells and then with their plasma membrane fractions. Excess unlabelled ligand was added and specific binding was determined with Scatchard analysis to calculate the Kd of binding and the # of binding sites/cell or /membrane protein. Specific, displaceable binding indicated a binding protein (receptor). The cell line with the highest maspin binding was used as source to isolate a maspin receptor. In year 02 we carried this approach further by identification and characterization of a maspin binding protein. Two approaches were used: Maspin was crosslinked to Sepharose 4B and an affinity column was made. The plasma membrane preparation from the breast carcinoma cell line exhibiting the highest maspin binding was extracted, iodinated by the lactoperoxidase method and added to the maspin affinity column. Controls included BSA cross-linked to Sepharose 4B or cross-linked Sepharose4B alone. The bound fraction was eluted with 0.2 M glycine HCl pH 3.5, immediately neutralized with 1.0M Tris/saline, lyophilized and run on a gel. Autoradiograms were used to identify a specific binding protein. Since maspin is a serpin, candidate receptor molecules included membrane associated proteinases such as the uPA/uPAR complex or MT-MMPs. Since maspin, in addition to inhibiting invasion also inhibits cell motility (which in itself could explain its inhibition of invasion), inhibition by binding to the handful of known motility-stimulating ligand-receptor complexes such as scatter factor/hepatocyte growth factor-c-met receptor, autocrine motility factor-receptor, autoxin-receptor, bFGF and its receptor, interleukin 6 and its receptor, integrins, and E cadherin was investigated by doing a simultaneous Western blot on the eluted material with antibodies to these different molecules. Since another possibility was that maspin was directly binding a negative regulator of cell motility rather than inhibiting a positive regulator, TGFβ-receptor and retinoic acid-receptor complexes, known negative regulators of cell motility were investigated by Western blotting. Recently investigators have shown that maspin binds to single stranded tissue plasminogen activator (ss t-PA); this molecule which is secreted could be a target for maspin action. However in the vast majority of the carcinoma and melanoma lines we studied where maspin exerted a pronounced suppressive effect on both motility and invasion, no ss t-PA was detected in these lines; hence maspin must be acting on a different target. Since both invasion and motility involve complex intracellular pathways, we hypothesize that maspin triggered a signal transduction pathway leading to inhibition of invasion/motility. For that reason we investigated by differential display and microarray analysis various genes whose expression levels were altered (up or down) by the actions of maspin. Computational analysis of these genes identified several candidate pathways of action. We have further analyzed this microarray analysis (expression profiling) in year 03.

The human myoepithelial cell lines / xenografts subjected to the present microarray analysis exhibited a unique gross and characteristic phase contrast appearance. Ultrastructural studies of the xenografts confirmed the presence of abundant extracellular matrix devoid of

murine stromal cells, inflammatory cells and endothelial cells (angiogenesis). These findings suggested that microarray analysis would reflect only human gene expression. Karyotype analysis of the transformed myoepithelial cell lines revealed only minimal deviations from a normal karyotype suggesting that they would serve as a suitable normal myoepithelial cell surrogate. Furthermore the same gene products could be detected within the transformed myoepithelial cells of the xenografts as within normal myoepithelial cells *in situ*. These findings suggested that subsequent microarray analysis would reflect the normal myoepithelial cell phenotype.

These myoepithelial cell lines / xenografts which had been derived from benign human myoepithelial tumors of diverse sources (salivary gland, breast and lung) exhibited a common mRNA expression profile which was indicative of a tumor suppressor phenotype.With hierarchical clustering, a significantly distinct grouping of the myoepithelial cell / xenograft samples were observed based on an analysis of 3200 genes. With a subsequent analysis based on a subset of 207 genes ($p<0.05$) even tighter grouping of the myoepithelial samples was observed. Within this subset of 207 genes was an excess of developmental genes, both over and underexpressed. Additionally filtered genes, hierarchically clustered and rank ordered, exhibited a 3 fold differential (either over or underexpressed) between the myoepithelial samples and the non-myoepithelial samples. Analysis of these clusters revealed increased expression in genes belonging to the classes of extracellular matrix proteins, angiogenic inhibitors and proteinase inhibitors and decreased expression of genes belonging to the classes of angiogenic factors and proteinases. Numerous expressed sequence tags also clustered within each of the aforementioned classes. Northern blot, Western blot and functional assays, eg. zymograms confirmed the increased / decreased expression of selected genes noted on microarray analysis.

2. Maspin in FNA

We have utilized maspin antibodies on FNA specimens to quantitate the myoepithelial component and determine if this determination discriminates between DCIS and invasive breast carcinoma. Polyclonal (rabbit) and monoclonal (murine) antibodies to maspin have been used (Pharmingen, San Diego, CA) according to standard immunocytochemical cytological protocols. We had great success with this approach in year 01 and expanded this to a much larger number of cases in year 02. We chose cases known by subsequent biopsy to be either pure DCIS or predominantly invasive carcinoma (most invasive breast cancers have at least some DCIS component). Without knowing the surgical pathology we determined first whether there were any myoepithelial cells present on the FNA and we then determined their number by assessing them by maspin positive immunostaining. We determined both the absolute number of myoepithelial cells and the ratio of myoepithelial cells to epithelial cells (the immunopositive to immunonegative ratio) and grouped the cases where there were no myoepithelial cells and the cases where there were some myoepithelial cells and determined the mean number $\pm$ standard deviation in this second group. We then subdivides these results into cases of known DCIS and cases of predominantly invasive cancer and did a t test on the results to see if DCIS and invasive breast cancer differed in their average number of myoepithelial cells present on FNA. Analyzing the results in this manner assumed and confirmed a Gaussian distribution for the number of myoepithelial cells in both groups. Based on the differences in the mean values and/or the slope of this curve we were able to perform power calculations to determine the number of cases needed to achieve statistical significance. Basically the steeper the slope of this curve the more discriminating the myoepithelial measurement

was and the fewer the number of cases needed. Based also on the differences in the mean values (how many standard deviations apart) of myoepithelial cell numbers or myoepithelial/epithelial ratios between our two groups, DCIS and invasive carcinoma, we were able also to perform power calculations and determine the number of cases needed. Furthermore we have used antibodies to smooth muscle actin, S100 and CALLA, all of which are fairly myoepithelial specific to compare with our maspin results. All of the latter antibodies recognized structural myoepithelial components and as such quantitate myoepithelial cell number. Maspin, on the other hand, not only identified myoepithelial cell number but provided an index of myoepithelial cell function---after all maspin is a serpin and a paracrine tumor suppressor. Therefore the use of maspin in this setting was more informative. In year 03 we further expanded on the number of case and began prospective studies.

3. Maspin in Ductal Fluid

Maspin in ductal fluid was measured by Western blot with total protein normalization. Human subjects approval for the collection of ductal fluid through select duct cannulation was obtained.. Small aliquots of this collected ductal fluid (50 patients) was used for the present studies. Nipple aspirates were collected by nipple suction (Sartorius/Petrakis). Analysis of nipple aspirates revealed the pooled contributions of all or most of the ducts where selective ductal cannulation allowed for a discrimination of one duct from another (eg, a duct with DCIS or microcalcifications *v* a normal duct. Our findings indicated that maspin was present reflecting the integrity of the ductal-lobular unit. We also demonstrated maspin in saliva, a fluid which reflects the abundance of myoepithelial cells in the salivary glands (See publication in Appendix).

In year 02, ductal fluid was begun to be collected following cannulation and washing of selected ducts in patients with microcalcifications on screening mammography who are about to undergo either excisional or core biopsy. In year 03, we will expand this to a larger number of cases. Paired comparisons of maspin levels in ductal fluid obtained from ducts harboring microcalcifications or DCIS and normal ducts from the same patients have and will continue to be made. Maspin levels will be correlated with the histopathology surrounding the microcalcifications. We have shown that some of these patients exhibit normal ductal histopathology surrounding their microcalcifications, some exhibit proliferations like hyperplasia, adenosis, ADH, and DCIS and still others invasive carcinoma. The screening value of maspin levels in all of these patients is emerging. Our approach to the evaluation of maspin values and to their discriminatory ability among the different groups has been similar to that given above for the analysis of myoepithelial cell number. Measurements of *myoepithelial* maspin in ductal fluid will continue to be compared to levels of a breast *epithelial* cell marker such as carcinoembryonic antigen (CEA) or bFGF. We, in fact, have recently found that bFGF is elevated in ductal fluid of certain patients. Hence the maspin/CEA or maspin/bFGF ratio might be predictive of risk with increased maspin/CEA or increased maspin/bFGF correlating with normalcy and decreased maspin/CEA or decreased maspin/bFGF correlating with either high risk, microcalcifications and/or precancerous histopathology.

In year 03 we focused more closely on this aim, expanding the number of cases and correlating the levels of maspin with the structural integrity of the ductal lobular unit. Our findings indicated that when the integrity of the ductal lobular unit is preserved the levels of maspin are not influenced by the degree of epithelial proliferation. The levels of other markers, eg. CEA, PSA, bFGF are however. When there is invasive cancer however and the myoepithelial

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APPENDIX

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Myoepithelial mRNA expression profiling reveals a common tumor-suppressor phenotype

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Abstract

A series of myoepithelial cell lines and xenografts derived from benign human myoepithelial tumors of diverse sources (salivary gland, breast, and lung) exhibit common mRNA expression profiles indicative of a tumor-suppressor phenotype. Previously established myoepithelial cell lines and xenografts (HMS-#; HMS-#X) were compared to nonmyoepithelial breast carcinoma cells (MDA-MB-231 and MDA-MB-468, and inflammatory breast carcinoma samples, IBCr, and IBCw), a normal mammary epithelial cell line (HMEC) and individual cases of human breast cancer (zcBT#T), and matched normal human breast tissues (zcBT#N) (overall samples = 22). The global gene expression profile (22,000 genes) of these individual samples was examined using Affymetrix Microarray Gene Chips and subsequently analyzed with both Affymetrix and DChip algorithms. The myoepithelial cell lines/xenografts were distinct and very different from the nonmyoepithelial breast carcinoma cells and the normal breast and breast tumor biopsies. Two hundred and seven specifically selected genes represented a subset of genes that distinguished ($P < 0.05$) all the myoepithelial cell lines/xenografts from all the other samples and which themselves exhibited hierarchical clustering. Further analysis of these genes revealed increased expression in genes belonging to the classes of extracellular matrix proteins, angiogenic inhibitors, and proteinase inhibitors and decreased expression belonging to the classes of angiogenic factors and proteinases. Developmental genes were also differentially expressed (either over or underexpressed). These studies confirm our previous impression that human myoepithelial cells express a distinct tumor-suppressor phenotype.

Keywords: Myoepithelial cells; Tumor suppression; Expression profile; Microarrays; Hierarchical clustering; Angiogenic inhibitors; Angiogenic factors; Proteinase inhibitors; Proteinases; Developmental genes

Introduction

It has become clear that cancer cells come under the influence of important paracrine regulation from the host microenvironment (Cavenee, 1993). Such host regulation may be as great a determinant of tumor cell behavior in vivo as the specific oncogenic or tumor-suppressor alterations occurring within the malignant cells themselves, and may be mediated by specific extracellular matrix molecules, matrix-associated growth factors, or host cells themselves (Liotta et al., 1991; Safarians et al., 1996). Both positive (fibroblast, myofibroblast, and endothelial cell) and negative (tumor-infiltrating lymphocyte and cytotoxic macrophage) cellular regulators exist which profoundly affect tumor cell behavior in vivo (Cornil et al., 1991; Folkman and Klagsbrun, 1987). One host cell, however, the myoepithelial cell, has escaped the paracrine onlooker's attention. The myoepithelial cell, which lies on the epithelial side of the basement membrane, is thought to contribute largely to both the synthesis and remodeling of this structure. This cell lies in juxtaposition to normally proliferating and differentiating epithelial cells in health and to abnormally proliferating epithelial cells in precancerous disease states. Myoepithelial cells also form a natural border separating epithelial cells from stromal angiogenesis. These anatomical relationships suggest that myoepithelial cells may exert important paracrine suppressive effects on epithelium and endothelium and may inhibit the progression of ductal carcinoma in situ (DCIS) to invasive breast cancer and carcinoma-induced

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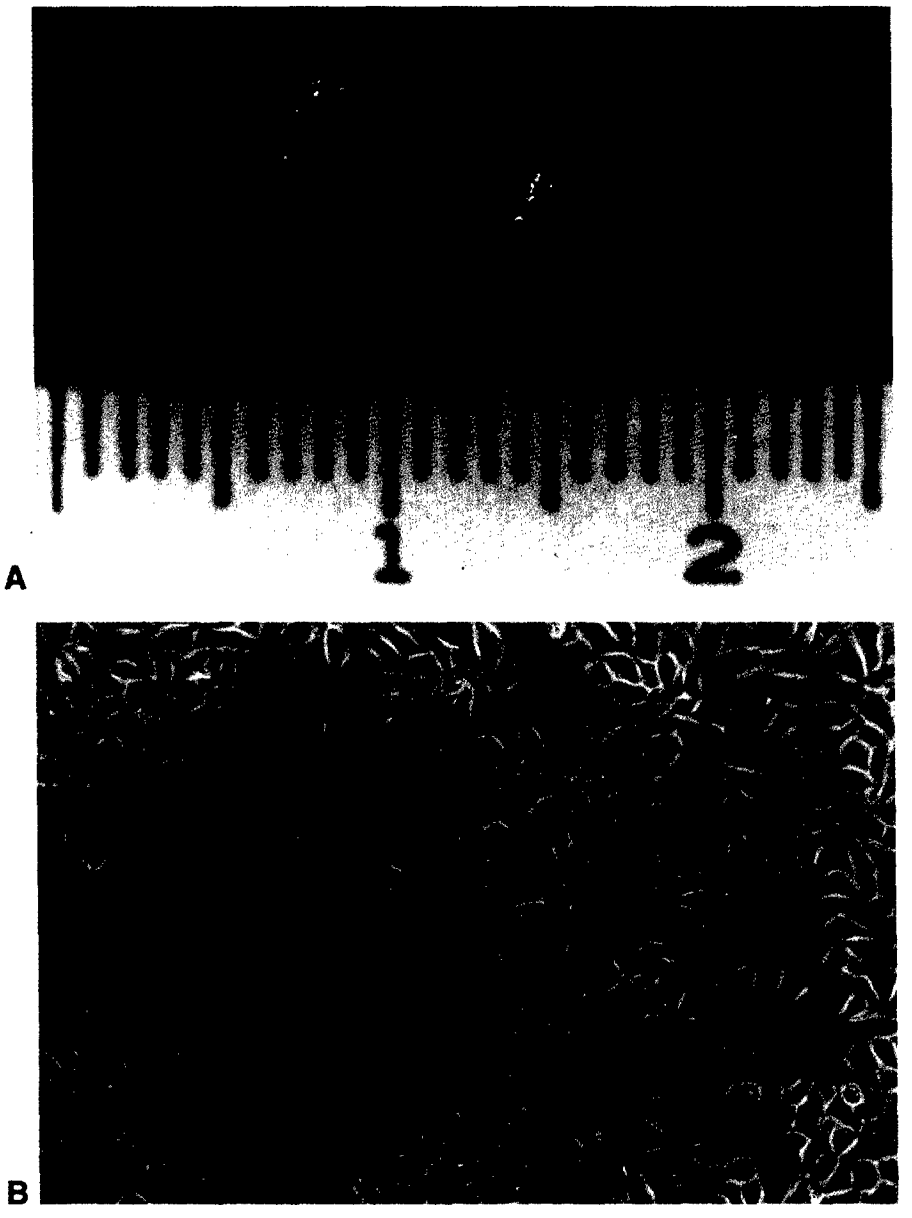
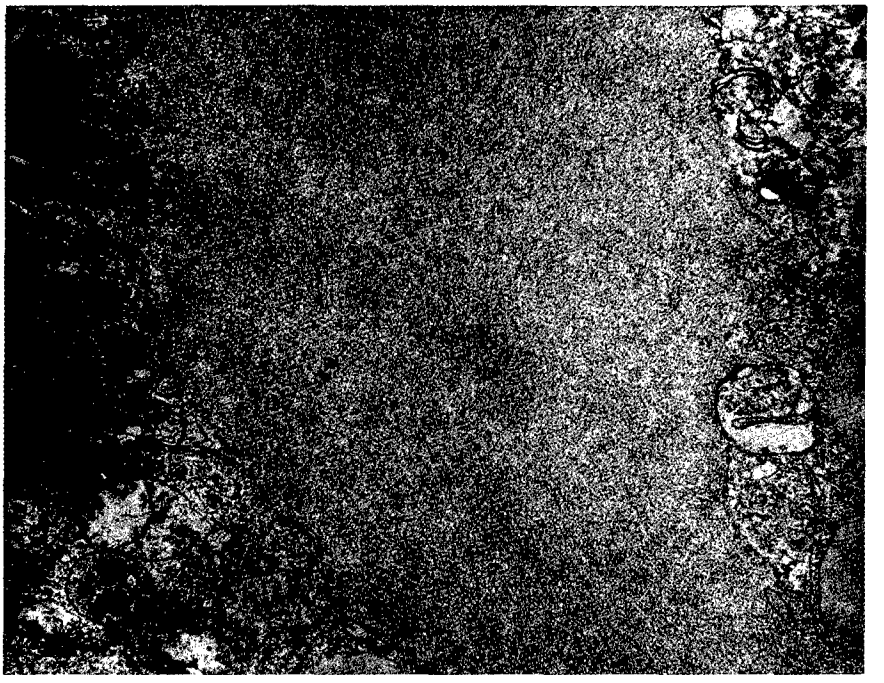


Fig. 1. (A) A typical myoepithelial xenograft, HMS-X, exhibits a white cartilaginous appearance due to the abundance of extracellular matrix; (B) a typical myoepithelial cell line, HMS-1, exhibits a polygonal cellular shape forming an epithelial-like monolayer; (C) ultrastructural studies of the xenograft, HMS-X, reveal a finely granular proteoglycan-containing extracellular matrix with absent fibroblasts, endothelial cells, and inflammatory cells; (D) karyotype of HMS-1 reveals a near normal diploid karyotype; (E) one gene product, maspin, was present immunocytochemically within the transformed myoepithelial cells of a typical myoepithelial xenograft, HMS-4X, and the same gene product was present within normal breast myoepithelial cells in situ (F). (A) Gross photograph; (B) phase contrast, ×200; (C) uranyl acetate, lead citrate, ×24,000; (D) karyotype with trypsin-Giemsa banding; (E) anti-maspin, immunoperoxidase, ×400; (F) anti-maspin, immunoperoxidase, ×400

angiogenesis. Additional evidence suggests that myoepithelial cells may also exhibit a natural autocrine suppressive phenotype. Myoepithelial cells rarely transform and when they do generally give rise to benign neoplasms and not malignant ones. There has been a paucity of studies on myoepithelial cells because they have been relatively difficult to culture and because tumors that arise from these cells are rare.

In previous studies we have successfully established immortalized cell lines and transplantable xenografts from benign or low-grade human myoepitheliomas of the salivary gland, breast, and lung (Barsky et al., 1988; Sternlicht et al., 1996, 1997). We collected these tumors in the 1980/s under IRB and NIH Human Subject Protection Committee Review Exemption 4 which applied to all excess human tissues removed for either diagnostic or therapeutic purposes which would otherwise be discarded. We also collected a number of nonmyoepithelial breast carcinomatous tissues including inflammatory breast cancers (IBCr and IBCw) during this same time period. Under Exemption 4, the identities of the human subjects were kept anonymous. The myoepithelial lines/xenografts collected were designated HMS-#; HMS-#X, respectively. These abbreviations stood for human ma-



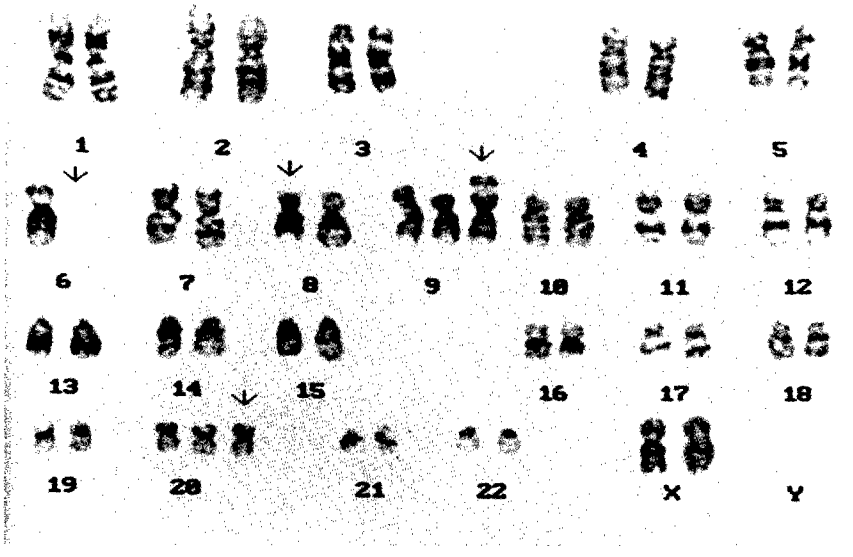


Fig. 1 (*continued*)

trix secreting line and xenograft, respectively, and referred to the chronological order of establishment. To date 4 lines/xenografts numbered HMS-1–6 and HMS-X–6X have been immortalized. HMS-2 and 2X and HMS-5 and 5X proved nonviable. The 4 established cell lines/xenografts displayed an essentially normal diploid karyotype and expressed identical myoepithelial markers as normal myoepithelial cells in situ. Unlike the vast majority of human tumor cell lines and xenografts which exhibited matrix-degrading properties these myoepithelial lines/xenografts like their myoepithelial counterparts in situ retained the ability to secrete and accumulate an abundant extracellular matrix. When grown as xenografts, the tumors were essentially avascular and their extracellular matrix was human and not murine in origin (Nguyen et al., 2000). When grown as a monolayer, one prototype myoepithelial cell line, HMS-1, exerted profound and specific effects on normal epithelial and primary carcinoma morphogenesis (Sternlicht et al., 1996). These studies supported our view that our established myoepithelial cell lines/xenografts could serve as primary myoepithelial cell surrogates. Because of our previous studies which suggested that human myoepithelial cells naturally exert a tumor-suppressive phenotype (Nguyen et al., 2000; Sternlicht et al., 1997), we decided to subject our human myoepithelial cell lines/xenografts to comparative mRNA expression profiling (gene chip analysis) to see whether the different myo-

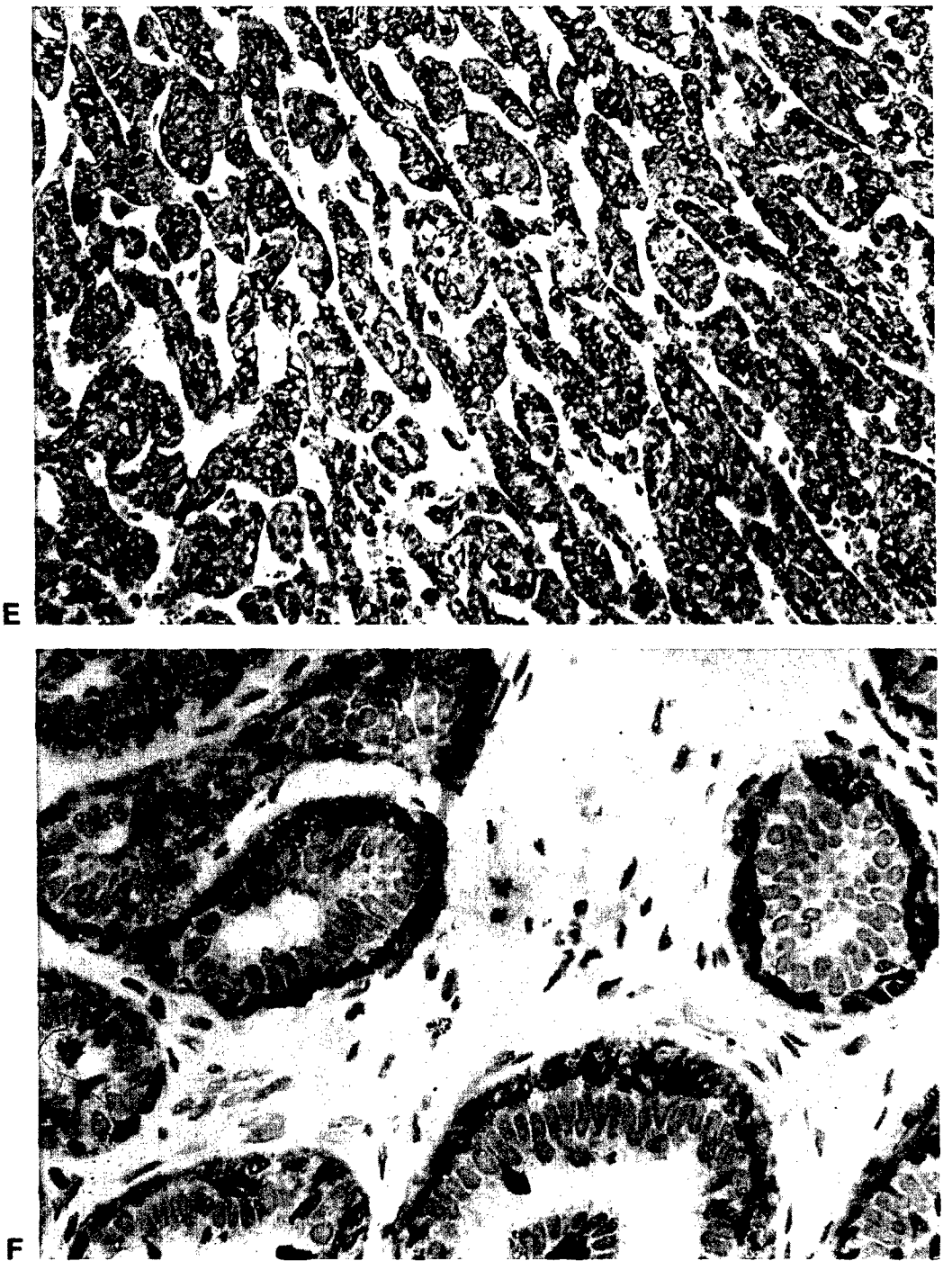


Fig. 1 (*continued*)

epithelial cell lines/xenografts group together and what gene clusters emerge.

Materials and methods

Source materials

Previously established myoepithelial cell lines and xenografts (HMS-1; HMS-X–HMS-6X) were subjected to standard gross, microscopic, ultrastructural, immunocytochemical, karyotype, and phase-contrast studies. These myoepithelial cell lines/xenografts were also compared to nonmyoepithelial breast carcinoma cells (MDA-MB-231, MDA-MB-468, IBCr, IBCw), a normal mammary epithelial cell line (HMEC) and individual cases of human breast cancer (zcBT#T), and matched normal human breast tissues (zcBT#N) (overall samples = 22).

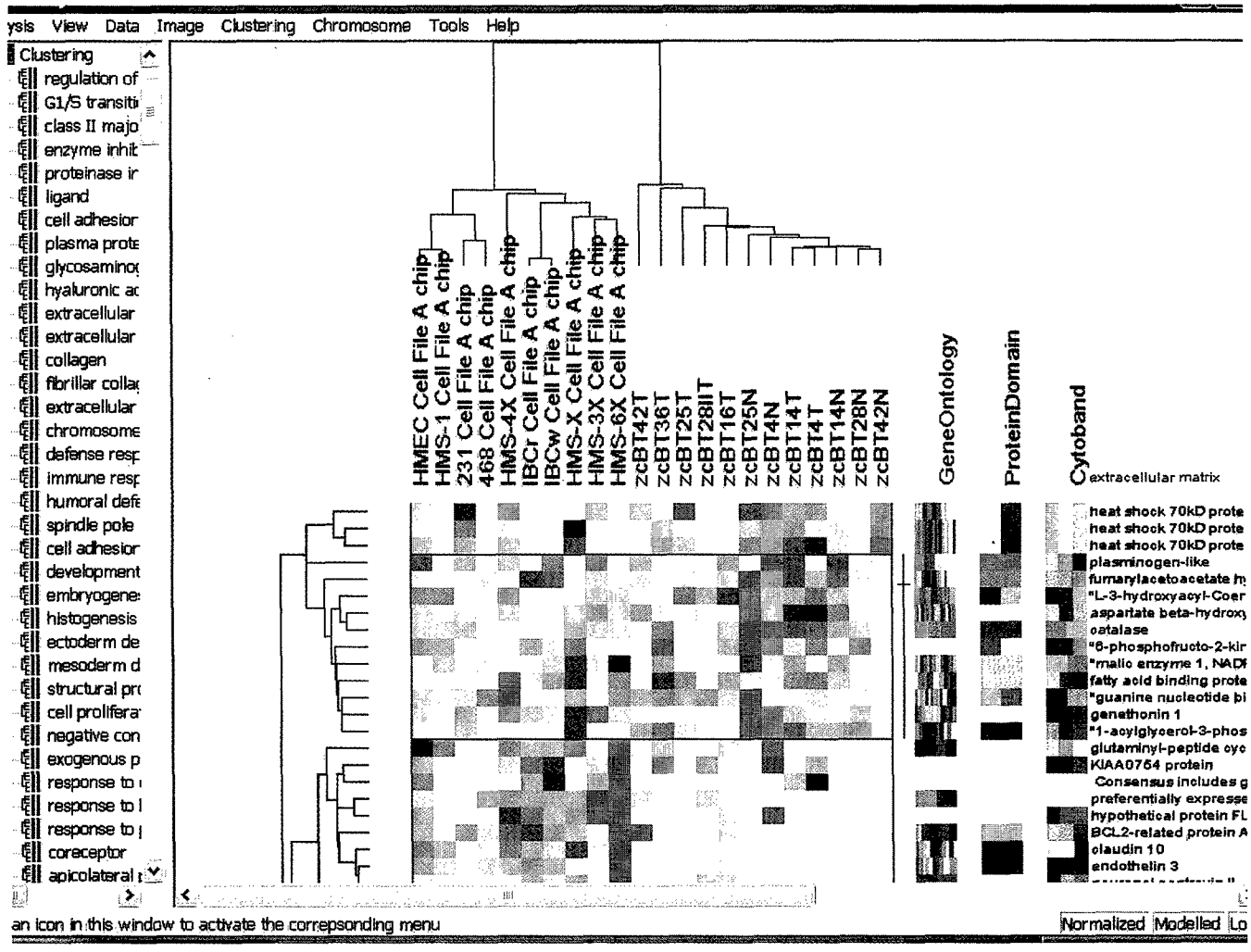


Fig. 2. Hierarchical clustering of 22 samples ("experiments") and 3200 genes that varied across all the experiments. Red indicates relative overexpression; blue, relative underexpression. The general pattern is that the myoepithelial samples are distinct from the nonmyoepithelial samples. Dendrogram, 3200 genes.

Hybridization and scanning

Ten micrograms of total RNA extracted from each sample was amplified by standard in vitro transcription methods and chromogen-labeled. Hybridization was carried out according to Affymetrix protocols and chips were scanned and gene intensities were recorded. All data were log 2-transformed with negative values set to 1. Ratios of gene expression were calculated by subtracting log 2 values.

Gene chip analysis

The expression levels were calculated by using DChip algorithms where individual sample expression was compared to the mean of all samples. Red indicated higher expression and green (or blue) indicated lower expression. Black indicated an expression level equal to the mean. Each gene chip analysis was termed an "experiment." The global gene expression profiles (22,000 genes) of these individual experiments were examined using Affymetrix Microarray Gene Chips and subsequently analyzed with both Affymetrix and DChip algorithms. An initial subset of 3200 genes that maximally varied among the experiments was selected. All the samples and all these genes were then subjected to hierarchical clustering. Based on the results of this initial analysis, a further subset of 207 genes representing genes which specifically distinguished ($P < 0.05$) the myoepithelial cell lines/xenografts from all the other samples and which themselves exhibited hierarchical clustering was used. The names and identities of the genes were recorded. Additionally filtered gene files, the subset of genes remaining after noninstructed forms of filtering, selected for genes that were varying across all the samples maximally. Genes with a 3-fold differential (either over or underexpressed) between the myoepithelial cell lines/xenografts and the nonmyoepithelial samples were rank-ordered. The classes of genes showing the highest fold differentials were recorded.

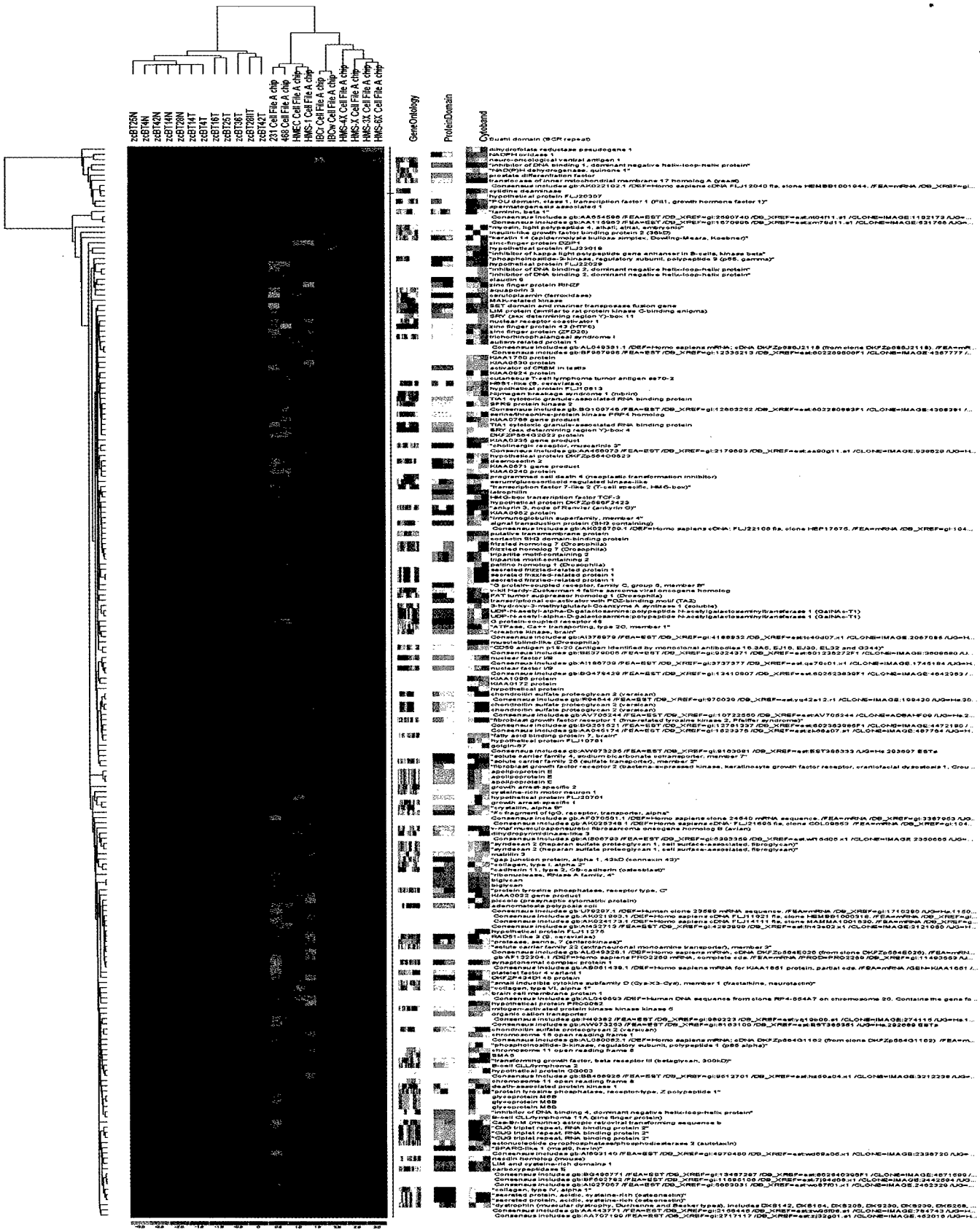


Fig. 3. Hierarchical clustering of 22 samples and 207 genes. Many of these genes were developmental genes, which represent the subset of genes which distinguish the myoepithelial samples from the nonmyoepithelial samples at a significance level of $P < 0.05$. The point of this dendrogram is to appreciate the checkerboard pattern of gene expression noted in the upper right corner of the dendrogram, which contributes to the hierarchical clustering of the myoepithelial samples into a distinct group:

Nonmyoepithelial	Myoepithelial
Red (overexpressed)	Green (underexpressed)
green (underexpressed)	Red (overexpressed)

Dendrogram, 3200 genes.

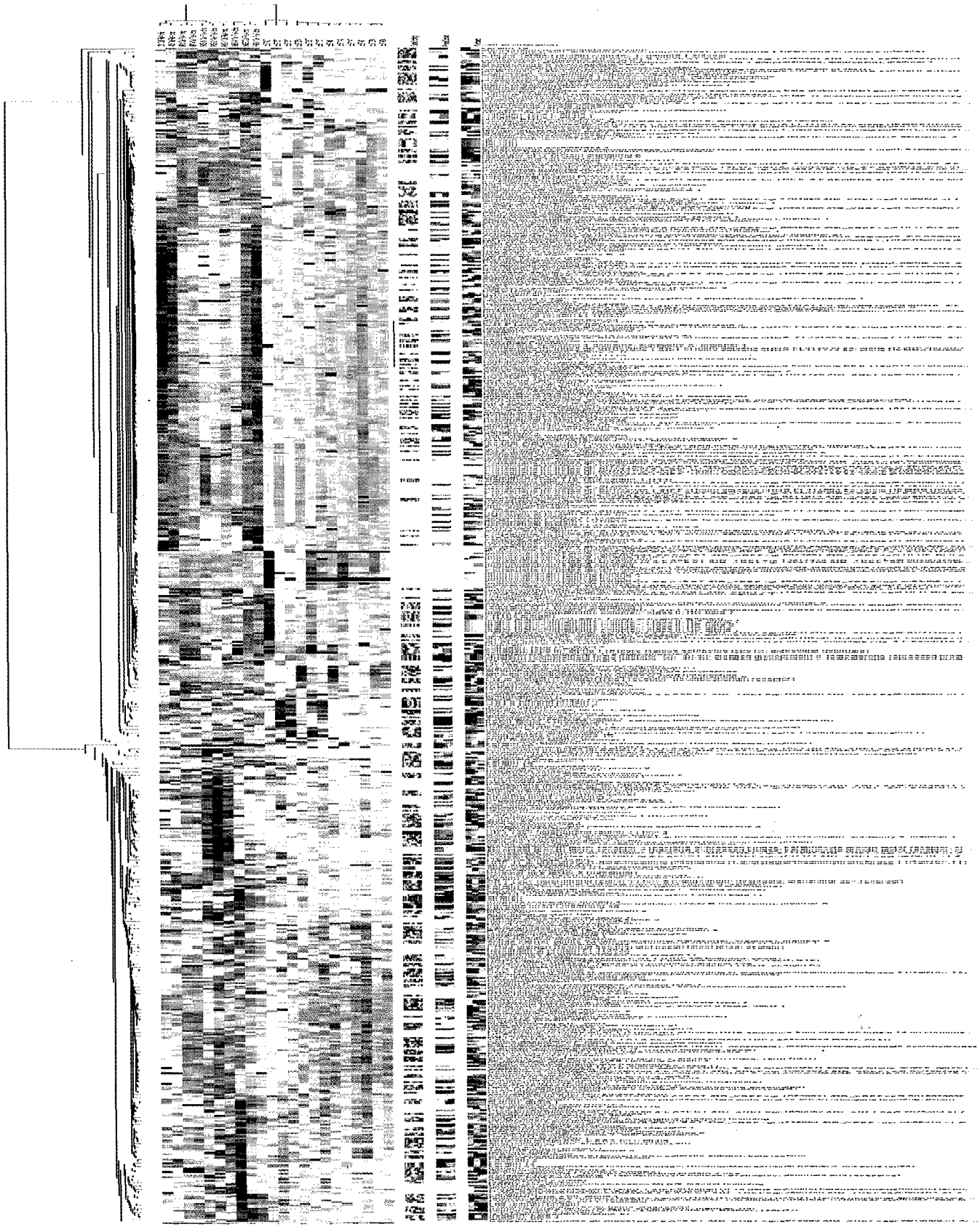


Fig. 4. Filtered genes are depicted which exhibit a 3-fold differential expression between the myoepithelial vs. nonmyoepithelial samples. In the upper left part of the dendrogram, myoepithelial underexpressed genes are depicted (blue); in the lower left part, myoepithelial overexpressed genes are depicted (red). The point of this dendrogram is not to depict the individual genes but rather the overall pattern of differential expression. Dendrogram, differentially expressed genes.

Table 1
Genes differentially expressed in myoepithelial vs non-myoepithelial cells

Class	Gene	Gene	Expression
Extracellular matrix			ALL ↑
	COL1A1	Laminin B	
	COL4A1	Nidogen/Entactin	
	COL4A2	BM90	
	HSPG2	Bamin	
	FN1	BM40/Osteonectin	
	Laminin A		
Proteinase inhibitors			ALL ↑
	Maspin	α1-AT	
	TIMP-1	31-kDa inhibitor	
	PN-II	PAI-1	
Proteinases			ALL ↓
	72-kDa gelatinase	Stromelysin	
	92-kDa gelatinase	uPA	
Angiogenic inhibitors			ALL ↑
	Thrombospondin-1	TIMP-1	
	Soluble bFGF receptors	Plasminogen	
	Plasminogen	Prolactin	
Angiogenic factors			ALL ↓
	bFGF	VEGF	
	aFGF	Angiogenin	
	TGFα	Platelet-derived ECG	
	TGFβ	Placental growth factor	
	TNFα	HB-ECGF	
	HGF		
Developmental genes			BOTH ⇅
	See Fig. 3		

Abbreviations used: COL1A1, type I collagen α1 chain; COL4A1, type IV collagen α1 chain; COL4A2, type IV collagen α2 chain; HSPG2, perlecan; FN1, fibronectin; TIMP-1, tissue inhibitor of metalloproteinase-1; PN-II, protease nexin-II; α1-AT, α1- antitrypsin; PAI-1, plasminogen activator inhibitor-1; uPA, urokinase-type plasminogen activator; bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor; TFGα, transforming growth factor α; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; platelet-derived ECGF, platelet-derived endothelial cell growth factor; HB-ECGF, heparin-binding endothelial cell growth factor.

Confirmation by Northern blot, Western blot, and zymograms

For select genes the findings on chip analysis were confirmed by either Northern blot, Western blot, or zymogram analysis according to established procedures.

Results

The human myoepithelial cell lines/xenografts subjected to the present microarray analysis exhibited a unique gross (Fig. 1A) and characteristic phase-contrast appearance (Fig. 1B). Ultrastructural studies of the xenografts confirmed the presence of abundant extracellular matrix devoid of murine stromal cells, inflammatory cells, and endothelial cells (angiogenesis) (Fig. 1C). These findings suggested that microarray analysis would reflect only human gene expression. Karyotype analysis of the transformed myoepithelial cell lines revealed only minimal deviations from a normal karyotype (Fig. 1D) suggesting that they would serve as a suitable normal myoepithelial cell surrogate. Furthermore the same gene products could be detected within the transformed myoepithelial cells of the xenografts (Fig. 1E) as within normal myoepithelial cells in situ (Fig. 1F). These findings suggested that subsequent microarray analysis would reflect the normal myoepithelial cell phenotype.

These myoepithelial cell lines/xenografts which had been derived from benign human myoepithelial tumors of diverse sources (salivary gland, breast, and lung) exhibited a common mRNA expression profile which was indicative of a tumor-suppressor phenotype (Figs. 2–5; Table 1). With hierarchical clustering, a significantly distinct grouping of the myoepithelial cell/xenograft samples was observed based on an analysis of 3200 genes (Fig. 2). With a subsequent analysis based on a subset of 207 genes ($P < 0.05$) even tighter grouping of the myoepithelial samples was observed (Fig. 3). Within this subset of 207 genes was an excess of developmental genes, both over and underexpressed (Fig. 3; Table 1). Additionally filtered genes, hierarchically clustered and rank ordered, exhibited a 3-fold differential (either over or underexpressed) between the myoepithelial samples and the nonmyoepithelial samples (Fig. 4). Analysis of these clusters revealed increased expression in genes belonging to the classes of extracellular matrix proteins, angiogenic inhibitors, and proteinase inhib-

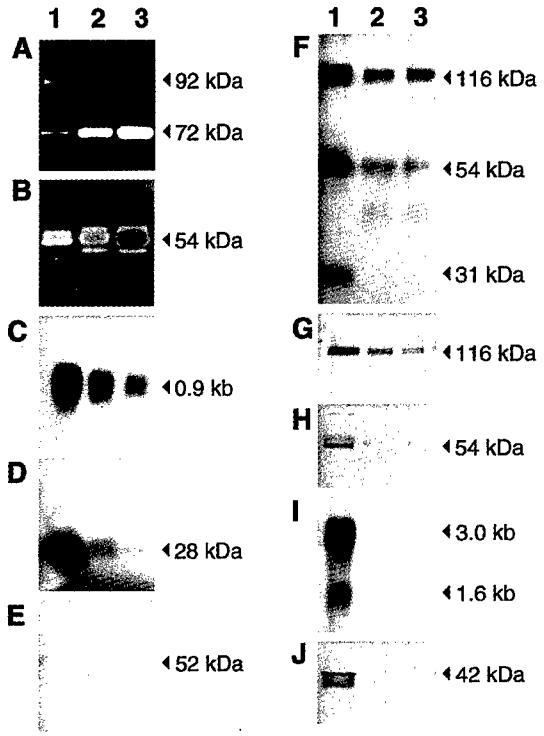


Fig. 5. Confirmatory pattern of expression of one prototype myoepithelial cell line, HMS-1 (lane 1), compared to two prototype nonmyoepithelial cell lines (lanes 2 and 3): direct gelatin zymography depicting the 92- and 72-kDa type IV collagenases confirms decreased expression in the myoepithelial cell line (A); direct fibrin zymography revealing the 54-kDa uPA also confirms decreased expression of this proteinase in the myoepithelial cell line (B); TIMP-1 expression reveals increased expression in the myoepithelial cell line by both Northern blot (C) and reverse zymography (D); another proteinase inhibitor, PAI-1, shows similarly increased expression in the myoepithelial cell line by reverse fibrin zymography as a 52-kDa lysis-resistant band (E); trypsin-like serine proteinase inhibitor expression is also increased in the myoepithelial cell line as demonstrated by reverse trypsin zymography as resistant bands of 116, 54, and 31 kDa (F); equivalently loaded Western blots using PN-II and α1-AT antibodies confirming increased expression in the myoepithelial cell line of two of the inhibitors: the 116-kDa PN-II (G) and the 54-kDa α1-AT inhibitor (H); the expression of maspin, a serine proteinase inhibitor and angiogenic inhibitor, is also dramatically increased in the myoepithelial cell line by Northern blot as 3.0- and 1.6-kb mRNA transcripts (I) and Western blot as a 42-kDa protein (J). See Table 1 for abbreviations used.

itors and decreased expression of genes belonging to the classes of angiogenic factors and proteinases (Table 1). Numerous expressed sequence tags also clustered within each of the aforementioned classes. Northern blot, Western blot, and functional assays, e.g., zymograms confirmed the increased/decreased expression of selected genes noted on microarray analysis (Fig. 5).

Discussion

It is the hope of high throughput approaches that the totality of gene expression or at least a subset of this totality will prove useful in hypothesis generation and eventual hypothesis testing. In the case of myoepithelial cells, the fact that diverse cell lines/xenografts derived from different benign neoplasms from disparate sites all cluster together supports the hypothesis that the myoepithelial phenotype is a distinct entity worthy of further study. When one considers the fact that myoepithelial cells rarely transform and when they do, transform to benignity and not malignancy (Guelstein et al., 1993), one could generate the following hypotheses: (1) that myoepithelial cells have an endogenous program of chemoprevention and (2) that myoepithelial cells have an endogenous program of metastasis suppression. Both of these programs would be contained in their pattern of global gene expression and perhaps in the subset of genes specifically characteristic of the myoepithelial phenotype (Zhang et al., 2000). One gene or the orchestrated expression of many genes may exert chemoprotective effects and similarly one gene or the orchestrated expression of many genes may suppress metastasis formation or contribute to tumor dormancy. Our microarray analysis employing hierarchical clustering demonstrated the strong grouping of the class of myoepithelial samples which clustered distinctly and very differently from nonmyoepithelial breast carcinoma cell lines, normal breast, and breast tumor biopsies. Hierarchical clustering of the genes involved revealed within the myoepithelial samples groups of genes belonging to the classes of extracellular matrix proteins, angiogenic inhibitors, and proteinase inhibitors, all of which showed increased expression, and groups of genes belonging to the classes of angiogenic factors and proteinases, all of which showed decreased expression. Numerous expressed sequence tags clustered with each of the aforementioned classes. When one considers the appearances of the myoepithelial xenografts, one is struck with their gross resemblance to cartilage. This appearance is due to the prominence of their extracellular matrix. One would obviously then anticipate that the class of genes which would show increased expression would be extracellular matrix genes and this was indeed the case. Myoepithelial tumors are prototype benign neoplasms which do not exhibit true invasion and which are devoid of any appreciable angiogenesis (Nguyen et al., 2000; Sternlicht et al., 1997). From these observations one would predict that they would exhibit increased expression of proteinase inhibitor and angiogenesis inhibitor genes and decreased expression of proteinases and angiogenic factor genes and this too was the case. One would not, however, predict that developmental genes would also be differentially expressed (either increased or decreased) in the myoepithelial samples. The hierarchical clustering of presently uncharacterized expressed sequence tags with each of the above-mentioned classes of genes suggests functional similarities and the possibility that they represent new angiogenic inhibitors or proteinase inhibitors which await discovery. When one considers that the myoepithelial phenotype is tumor suppressive and that myoepithelial cells have a built in chemoprevention and metastasis-suppression program, one could envision using this high

throughput gene expression profiling approach to identify candidate genes and candidate patchways which are either chemopreventive or metastasis suppressive in nature.

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10

Myoepithelium: Methods of Culture and Study

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1. INTRODUCTORY REVIEW

Paracrine regulation of tumor progression by host cells is an important determinant of tumor growth, invasion, and metastasis. However, one cell that has largely been ignored in this regulation is the myoepithelial cell. In any organ where there is significant branching morphogenesis, such as the breast, myoepithelial cells ubiquitously accompany and surround epithelial cells and are thought to keep in check (negatively regulate) the process of branching. Myoepithelial cells surround both normal ducts and precancerous lesions, especially of the breast (so-called DCIS, ductal carcinoma in situ), and form a natural border separating proliferating epithelial cells from proliferating endothelial cells (angiogenesis). Myoepithelial cells, by forming this natural border, are thought to negatively regulate tumor invasion and metastasis. Whereas epithelial cells are susceptible targets for transforming events leading to cancer, myoepithelial cells are resistant. Indeed tumors of myoepithelial cells are uncommon and, when they do occur, are almost always benign. Therefore, it can be said that myoepithelial cells function as both autocrine as well as paracrine tumor suppressors.

Our laboratory has found that myoepithelial cells secrete numerous suppressor molecules: high amounts of diverse proteinase inhibitors, which include maspin, TIMP-1, protease nexin-II, and α-1 antitrypsin; low amounts of proteinases and high amounts of diverse angiogenic inhibitors, which include maspin, thrombospondin-1, and soluble bFGF receptors, but low amounts of angiogenic factors compared with common malignant cell lines. Whereas carcinoma cells secrete more proteinases than proteinase inhibitors and more angiogenic factors than angiogenic inhibitors, myoepithelial cells then do just the opposite. This observation holds in vitro, in mice and in humans, and suggests

that myoepithelial cells exert pleiotropic suppressive effects on tumor progression.

This constitutive gene expression profile of myoepithelial cells may largely explain the pronounced anti-invasive and anti-angiogenic effects of myoepithelial cells on carcinoma and pre-carcinoma cells and may also account for the low-grade biology of myoepithelial tumors, which are devoid of appreciable angiogenesis and invasive behavior. Many of the secretory gene products of myoepithelial cells are present in body fluids, such as in breast ductal fluid and in saliva, reflecting the structural and functional integrity of the ductal-lobular units of the mammary and salivary glands, respectively. Some of the myoepithelial gene products; for example, maspin, in ductal fluid may serve as a surrogate (intermediate) end-point marker (SEM) to estimate the risk of DCIS progression to invasive cancer in the breast and, alternatively, in saliva, may serve as a tumor marker to detect the presence of incipient myoepithelial tumors occurring within the salivary glands of the head and neck.

In order to study the cell and molecular biology of myoepithelial cells further, methods to isolate and characterize human myoepithelial cells must be devised and perfected. Two sources of human myoepithelial cells are 1) the normal ductal-lobular units of organs rich in myoepithelial cells, such as the breast and salivary glands; and 2) benign tumors of myoepithelial cells where large numbers of myoepithelial cells, albeit transformed, can be obtained and used as normal myoepithelial cell surrogates.

2. STUDIES OF MYOEPITHELIAL CELLS

It has become clear that cancer cells come under the influence of important paracrine regulation from the host microenvironment [Cavenee, 1993]. Such host regulation may be as great a determinant of tumor cell behavior in vivo as the specific oncogenic or tumor suppressor alterations occurring within the malignant cells themselves, and may be mediated by specific extracellular matrix molecules, matrix-associated growth factors, or host cells themselves [Liotta et al., 1991; Safarians et al., 1996]. Both positive (fibroblast, myofibroblast, and endothelial cell) and negative (tumor-infiltrating lymphocyte and cytotoxic macrophage) cellular regulators exist, which profoundly affect tumor cell behavior in vivo [Folkman and Klagsbrun, 1987; Cornil et al., 1991]. However, one host cell, the myoepithelial cell, has escaped the paracrine onlooker's attention. The myoepithelial cell, which lies on the epithelial side of the basement membrane, is thought to contribute largely to both the synthesis and remodeling of this structure. This cell lies in juxtaposition to normally proliferating and

differentiating epithelial cells in health and to abnormally proliferating and differentiating epithelial cells in precancerous disease states, such as DCIS of the breast. This anatomical relationship suggests that myoepithelial cells may exert important paracrine effects on normal glandular epithelium and may regulate the progression of DCIS to invasive breast cancer. Circumstantial evidence suggests that the myoepithelial cell naturally exhibits a tumor suppressive phenotype. Myoepithelial cells rarely transform, and, when they do, they generally give rise to benign neoplasms that accumulate rather than degrade extracellular matrix [Guelstein et al., 1993]. Myoepithelial cells directly or indirectly through their production of extracellular matrix and proteinase inhibitors, including maspin, are thought to regulate branching morphogenesis that occurs in the developing breast and salivary gland during embryological development [Cutler, 1990]. There have been a paucity of studies on myoepithelial cells because they have been relatively difficult to culture and because tumors that arise from these cells are rare.

In previous studies we have been extremely fortunate to have successfully established immortalized cell lines and transplantable xenografts from benign or low-grade human myoepitheliomas of the salivary gland and breast [Sternlicht et al., 1996; Sternlicht et al., 1997]. These lines/xenografts have been designated HMS-#; HMS-#X, respectively. These pneumonics stand for human matrix secreting line and xenograft, respectively and refer to the chronological order of establishment. To date six lines/xenografts numbered HMS-1–6 and HMS-X–6X have been established. These lines and xenografts are available to investigators on request. These cell lines and xenografts displayed an essentially normal diploid karyotype and expressed identical myoepithelial markers as their in situ counterparts, including high constitutive expression of maspin. Unlike the vast majority of human tumor cell lines and xenografts, which exhibited matrix-degrading properties, these myoepithelial lines/xenografts, like their myoepithelial counterparts in situ, retained the ability to secrete and accumulate an abundant extracellular matrix composed of both basement membrane and non-basement membrane components. When grown as a monolayer, one prototype myoepithelial cell line, HMS-1, exerted profound and specific effects on normal epithelial and primary carcinoma morphogenesis [Sternlicht et al., 1996]. These studies support our position that our established myoepithelial lines/xenografts recapitulate a normal differentiated myoepithelial phenotype and can therefore be used experimentally as a primary myoepithelial cell surrogate. Prompted by these studies and by the conspicuous absence of studies examining the role of the myoepithelial cell in tumor progression, we decided to examine the myoepithelial cell from this perspec-

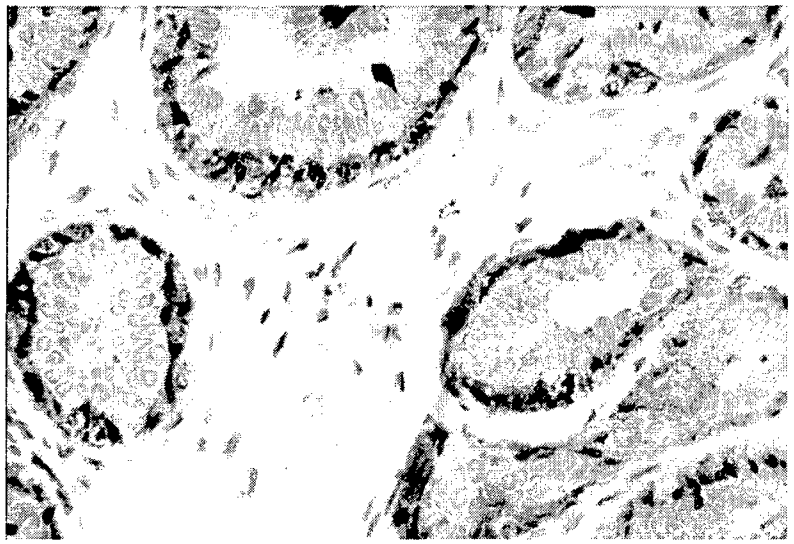
tive. Experiments with these cell lines/xenografts together with relevant in situ observations form the cornerstone of our studies, which observe that the human myoepithelial cell is a natural tumor suppressor.

3. INHIBITION OF TUMOR INVASION

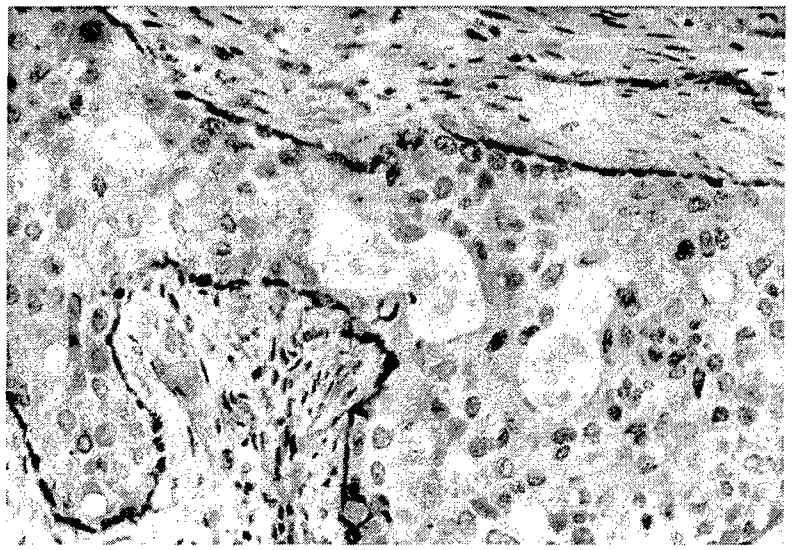
Breast ducts and acini are surrounded by a circumferential layer of myoepithelial cells exhibiting strong immunoreactivity for S100, smooth-muscle actin, CALLA, calponin, and diverse proteinase inhibitors, including maspin, α1-AT, PNII/APP, and TIMP-1 (Fig. 10.1A). In DCIS, the myoepithelial layer appeared either intact or focally disrupted, but the myoepithelial cells themselves exhibited the same pattern of immunoreactivity (Fig. 10.1B). In DCIS, although proliferations of vWf immunoreactive blood vessel capillaries were observed focally within the supporting stroma, such blood vessels were not observed within the proliferating DCIS cells on the epithelial side of the myoepithelial layer (Fig. 10.1C). The human tumoral-nude mouse xenografts derived from the human myoepitheliomas of the salivary gland, HMS-X and HMS-3X, and breast, HMS-4X demonstrated immunocytochemical profiles identical to each other and to that exhibited by the myoepithelial cells surrounding normal ducts and DCIS with especially intense maspin immunoreactivity (Fig. 10.1D).

Not only was strong proteinase inhibitor immunoreactivity present within the myoepithelial cells of these xenografts, but strong proteinase inhibitor immunoreactivity could be demonstrated within their extracellular matrix as well. Due to this matrix, the myoepithelial xenografts appeared white and cartilaginous in nature (Fig. 10.1E). Within this abundant extracellular matrix deposited by the different human myoepithelial xenografts, murine blood vessels were not observed (Fig. 10.1F). Through the use of a mouse-specific Cot-1 DNA probe (Fig. 10.1G), human myoepithelial xenografts HMS-X, HMS-3X, and HMS-4X demonstrated absent or near-absent angiogenesis. Human non-myoepithelial xenografts of breast cancer cell lines MDA-MB-231 and MDA-MB-468, in contrast, showed a comparatively large murine component of angiogenesis. In a two-dimensional matrix, myoepithelial cell lines grew as a confluent monolayer with self-forming spheroids at superconfluency (Fig. 10.1H). In a three-dimensional matrix, myoepithelial cell lines branched and budded (Fig. 10.1I). In monolayer culture, ultrastructural studies confirmed the cells' myoepithelial identity (Fig. 10.1J).

Detailed studies [Sternlicht et al., 1997] conducted with HMS-1, a prototype myoepithelial cell line, revealed a constitutively high proteinase inhibitor to proteinase ratio in strong contrast to the high

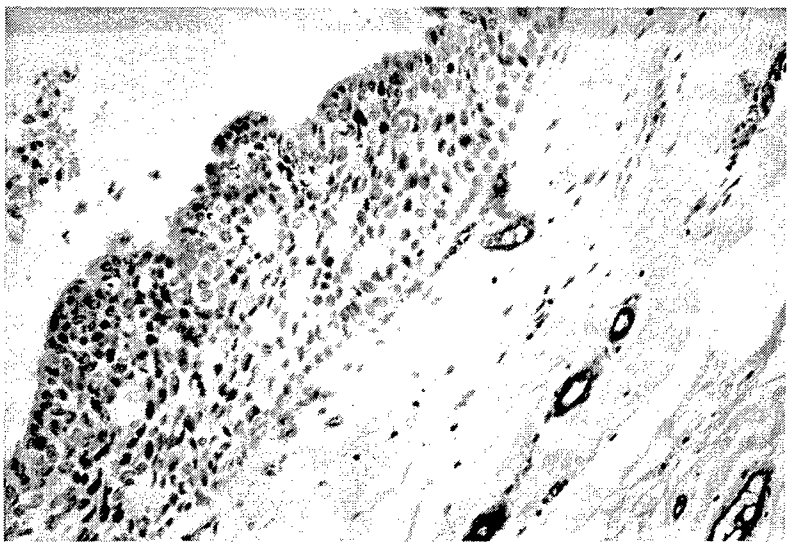


A



B

Figure 10.1. *In situ* immunocytochemistry profile of myoepithelial cells and their derived cell lines/xenografts. A) Differential maspin immunoreactivity of myoepithelial cells surrounding breast ducts and acini. B) Differential maspin immunoreactivity of myoepithelial cells in DCIS. C) Angiogenesis demonstrated by vWf immunoreactivity limited to stromal side of DCIS. D) Cytoplasmic maspin immunoreactivity of myoepithelial xenograft, HMS-X. E) Gross appearance of one myoepithelial xenograft, HMS-X. F) Microscopic appearance of one typical myoepithelial xenograft, HMS-4X, devoid of apparent angiogenesis. G) Murine Cot-1 dot blot. With a mouse-specific Cot-1 DNA probe, human myoepithelial xenografts, HMS-X, HMS-3X,



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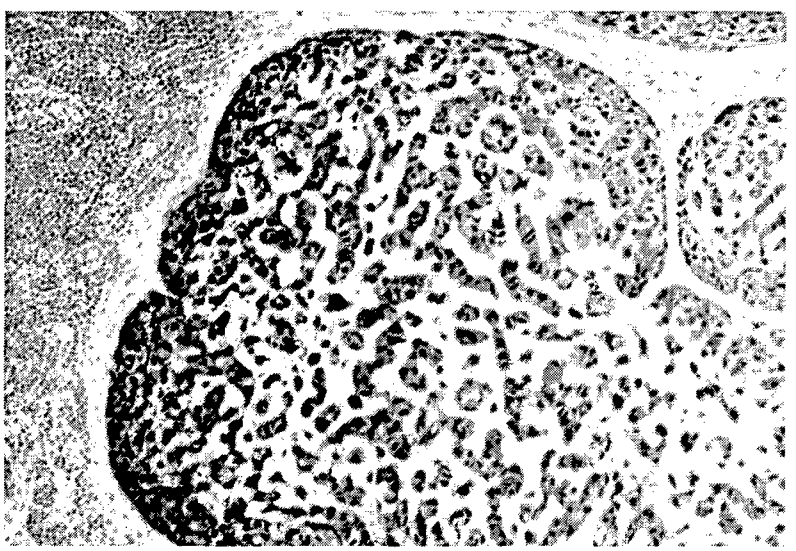
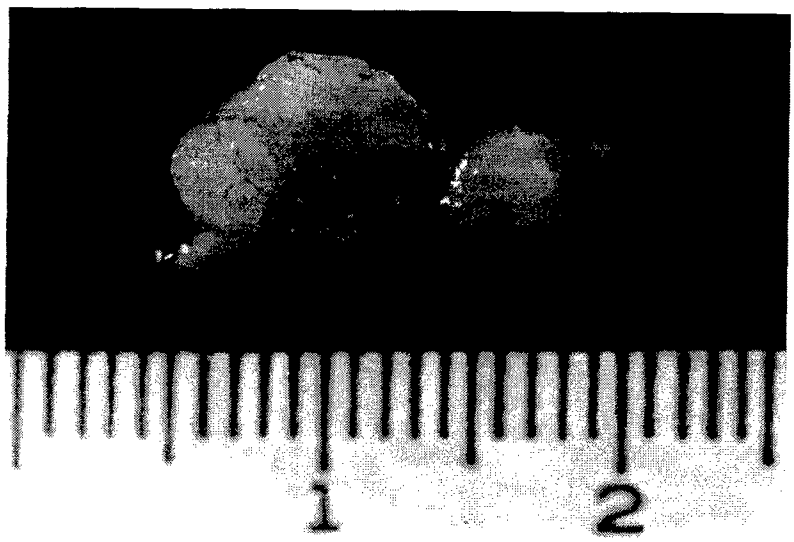


D

Figure 10.1. *(Continued)*
and HMS-4X are devoid of a murine DNA angiogenic component in contrast to the angiogenic-rich MDA-MB-231-X and MDA-MB-468-X breast carcinoma xenografts (right column); control dot blots of varying murine DNA percentages are also depicted (left column). H) The prototype myoepithelial cell line, HMS-1, in culture, grows as self-inducing spheroids on top of its own monolayer. I) HMS-1 undergoes branching morphogenesis and budding when grown on either Matrigel or a myoepithelial-derived matrix, *Humatrix.* J) Myoepithelial cells in culture, ultrastructurally, give the impression of "smiling," which is symbolic of the cells' natural tumor-suppressive function.



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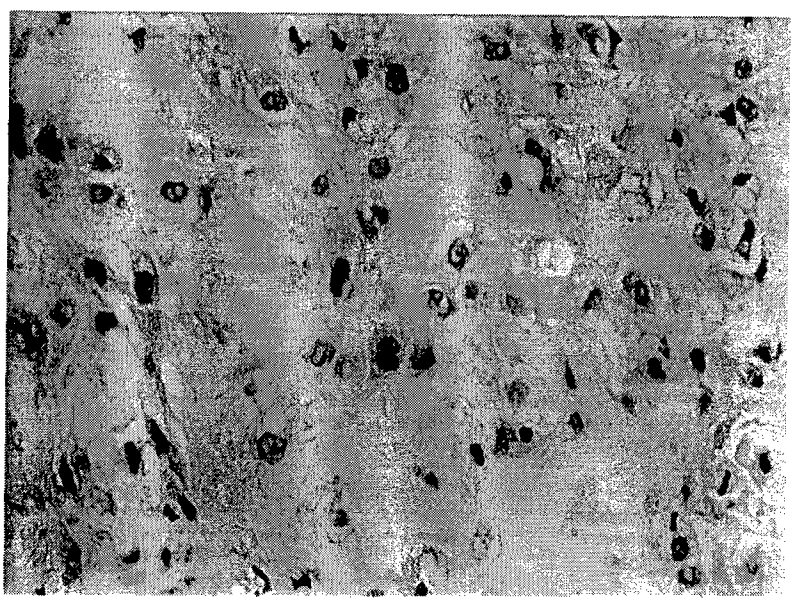

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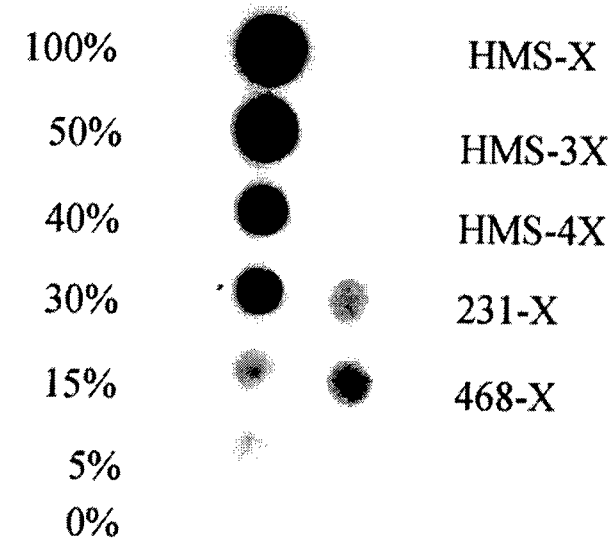
Figure 10.1. *(Continued)*



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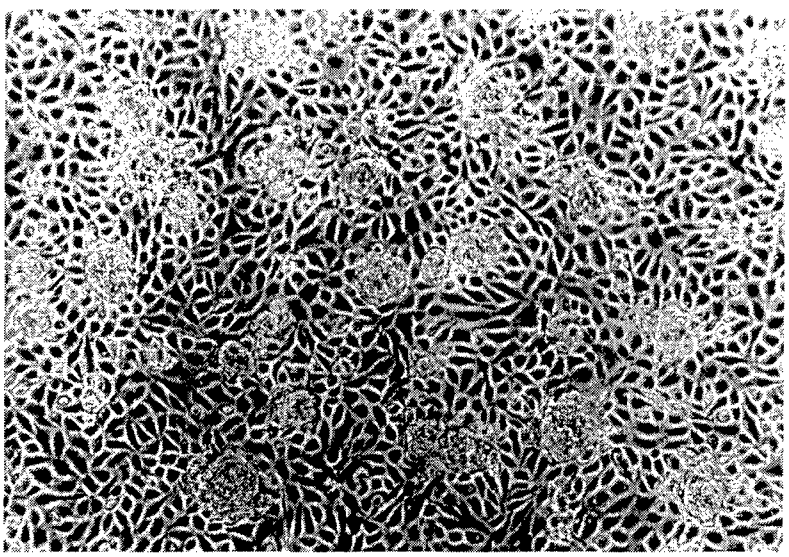

H

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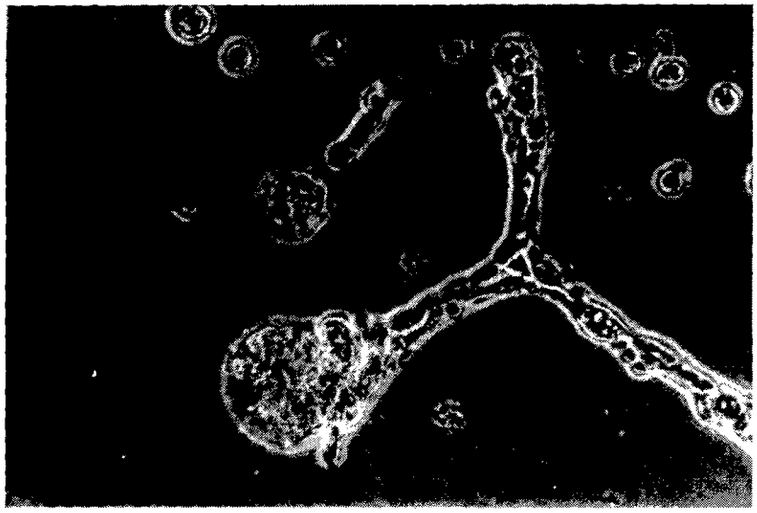

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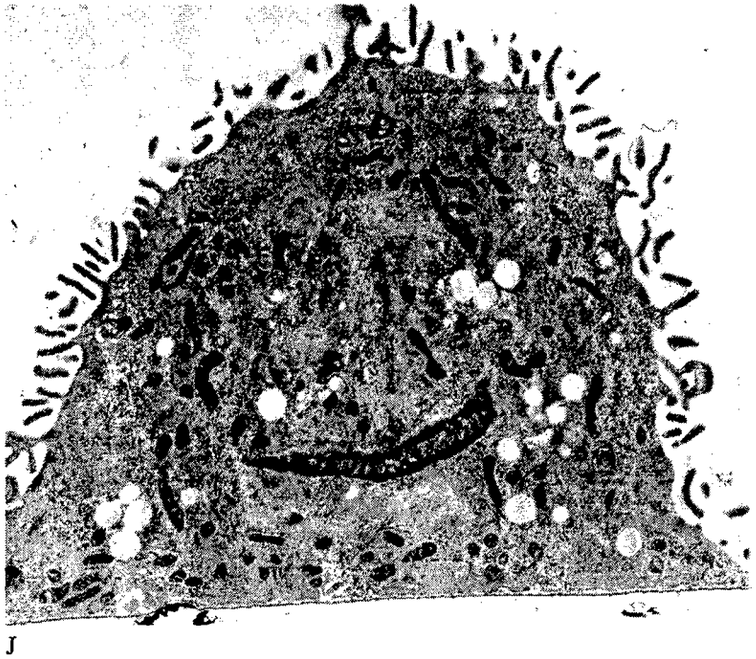


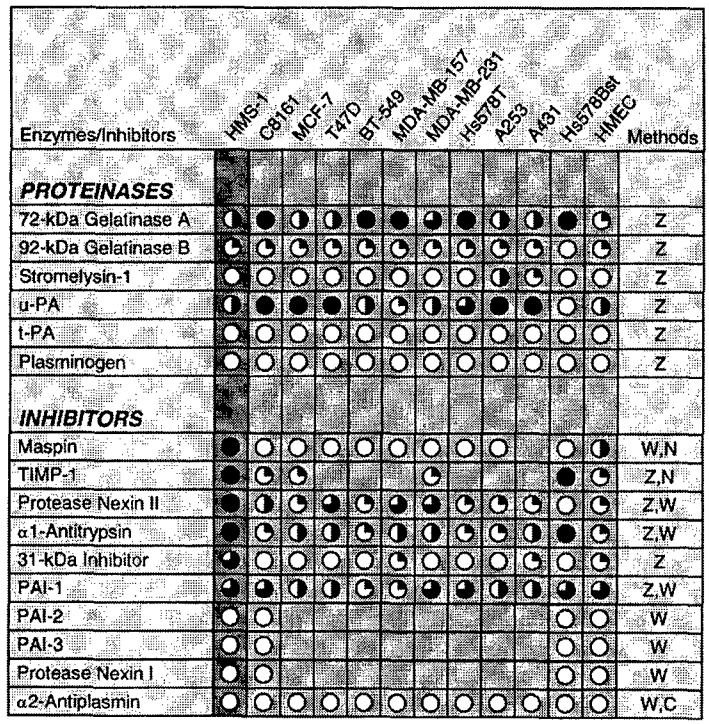
J

Figure 10.1. *(Continued)*

proteinase to proteinase inhibitor ratio observed in a number of malignant human cell lines (Fig. 10.2A). Marker studies with this cell line and corresponding xenograft (HMS-X) reflected the constitutive gene expression profile of myoepithelial cells in situ (Fig. 10.2B). This finding was especially true with respect to maspin. Direct gelatin zymography of conditioned media (CM) revealed only low levels of the 92 and 72 kDa type IV collagenases (MMP-9 and MMP-2, respectively) in HMS-1; the 72 kDa collagenase was reduced sixfold in HMS-1 compared with the levels in the majority of the malignant lines; direct fibrin zymography revealed visibly lower levels of the 54 kDa urokinase plasminogen activator (uPA) in HMS-1. This condition was also observed in casein/plasminogen gels.

Tissue-type plasminogen activator was not detected in any cell line, nor was plasmin detected in control gels lacking plasminogen. Stromelysin-1 (MMP-3) was also not detected in HMS-1. The proteinase inhibitor expression profile of HMS-1, in contrast, was characterized by high constitutive expression in CM of several proteinase inhibitors, including TIMP-1; PAI-1; three trypsin inhibitors: α1-AT, PNII/APP, and an unidentified 31 kDa inhibitor detected initially on reverse zymography; and the tumor suppressor maspin. With respect to the trypsin serine proteinase inhibitors, the conspicuous doublet at 116 kDa consistently greater in HMS-1 than in any of the other lines examined was confirmed on Western blot as PNII/APP. These bands represented the 770 and 751 amino acid isoforms of PNII/APP, which possessed a Kunitz-type serine proteinase inhibitor domain.

Interestingly, in 2 M urea extracts of HMS-X, HMS-3X, and HMS-4X, a novel 95 kDa band of trypsin inhibition was detected by reverse-zymography and was confirmed by Western blot to represent an active breakdown product of PNII. This 95 kDa PNII breakdown product was completely absent from HMS-1 CM and urea extracts of HMS-1 cells, suggesting that it was produced in situ within the myoepithelial extracellular matrix to which it bound. The retention of proteinase inhibitor activity by this breakdown product indicated that it retained the Kunitz-type serine proteinase inhibitor domain responsible for its ability to inhibit trypsin. In contrast to PNII/APP, protease nexin I was not detected. The second trypsin serine proteinase inhibitor was present at 54 kDa and was α1-AT. This inhibitor appeared nearly equivalent in HMS-1 compared with the malignant lines examined on reverse-zymography, but by Western blot its signal was markedly stronger and slightly more mobile in HMS-1 than in the malignant lines. These data were reconciled with the fact that α1-AT was probably less glycosylated in HMS-1. This relative underglycosylation caused α1-AT from HMS-1 to migrate slightly further into the gel and accounted for its poorer reactivation following sodium dodecyl sulfate

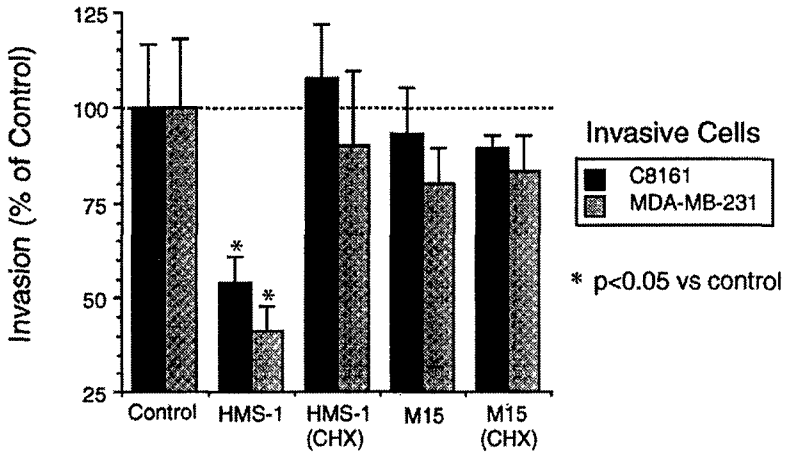


A

	HMS-X,3X,4X		Normal Breast		DCIS	
	Cells	Matrix	ME†	Epi‡	ME	Epi
S-100	++++*	-	++++	-	++++	-
Maspin	++++	-	++++	+	++++	±
α1-AT	++	++	++	-	++	-
PNII	++	+++	++	-	++	±
TIMP-1	++	+	++	-	++	-
PAI-1	+	±	+	+	+	+
vWf	-	-	-§	-§	-§	-§

B

Figure 10.2. A) Relative constitutive expression of diverse proteinase inhibitors and proteinases in myoepithelial cells (HMS-1) compared with various malignant cell lines. Z, direct or reverse zymography; W, Western blot; N, Northern blot; C, chromogenic substrate assay. B)



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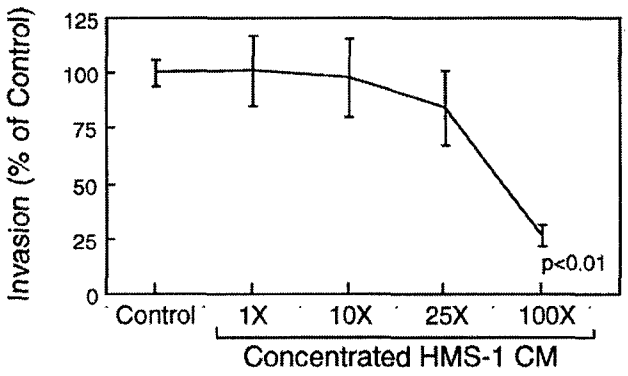
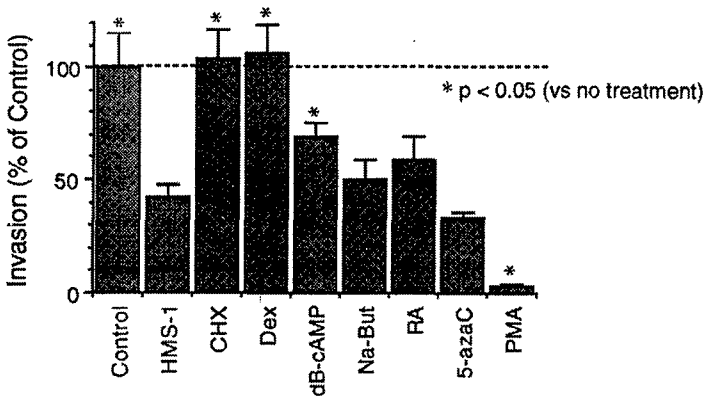


D

Figure 10.2. *(Continued)*
Myoepithelial-related immunoreactivity *In Situ.* [†]myoepithelial cells; [‡]epithelial cells; *++++, intensely positive; +++, strongly positive; ++, positive; +, weakly positive; ±, equivocally positive; −, negative; [§]on epithelial side of basement membrane. C) Effects of HMS-1 cells on C8161 (melanoma) and MDA-MB-231 (breast carcinoma) invasion. D) Effects of HMS-1 CM on invasion of C8161 cells. Results with MDA-MB-231 cells were similar. Assays in both (C, D) were performed in quadruplicate and show mean percentage of control invasion ± standard deviation. E) Effects of pharmacological treatment of HMS-1 cells with various agents inducing invasion-permissive and non-permissive phenotypes: CHX, cycloheximide; DEX, dexamethasone; dB-cAMP, N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate; Na-But, sodium butyrate; RA, all *trans* retinoic acid; 5-azaC, 5-azacytidine; PMA, phorbol 12-myristate 13-acetate. Dexamethasone induced invasive-permissive phenotype, whereas PMA induced a non-permissive phenotype. Invasion (percentage of control) of C8161 melanoma cells is depicted.



E

Figure 10.2. *(Continued)*

(SDS)-denaturation on reverse-zymography compared with the more highly glycosylated isoforms present in the malignant lines.

The third trypsin serine proteinase inhibitor detected at 31 kDa was clearly not a degradation product of either PNII or α1-AT, as demonstrated by negative Western blot. The 31 kDa inhibitor was strongly expressed in HMS-1 and was either absent or nearly absent in all of the malignant lines examined. It is being determined whether this unidentified inhibitor is a novel inhibitor. In contrast to the above inhibitors, PAI-1 was expressed only slightly greater in HMS-1 compared with the majority of the malignant lines by both reverse-zymographic and Western blot analysis. Neither PAI-2, PAI-3 or α2-antiplasmin were detected by Western blot analysis in any of the cell lines. Antiplasmin activity, as determined by photometric assay, was completely absent as well.

The most striking difference, however, between the strong proteinase inhibitor profile of HMS-1 and the profile of the malignant cell lines examined was in the expression of maspin. Intense maspin transcripts (3.0 and 1.6 kb) and protein (42 kDa) were identified in HMS-1 and HMS-1 CM, respectively, but were completely absent in all of the malignant lines examined (Fig. 10.2A). With its proteinase inhibitor profile of increased maspin, TIMP-1, PN-II, α1-AT, and the 31 kDa inhibitor, HMS-1 bore strong resemblance to normal human mammary epithelial cells (HMEC, Clonetics) (Fig. 10.2A) except that the expression of all of these proteinase inhibitors, including maspin, was even more enhanced in HMS-1. Being derived from normal ducts and acini of the human breast, HMEC cultures likely contain myoepithelial as well as epithelial cells. Thus, the resemblance of HMS-1 to

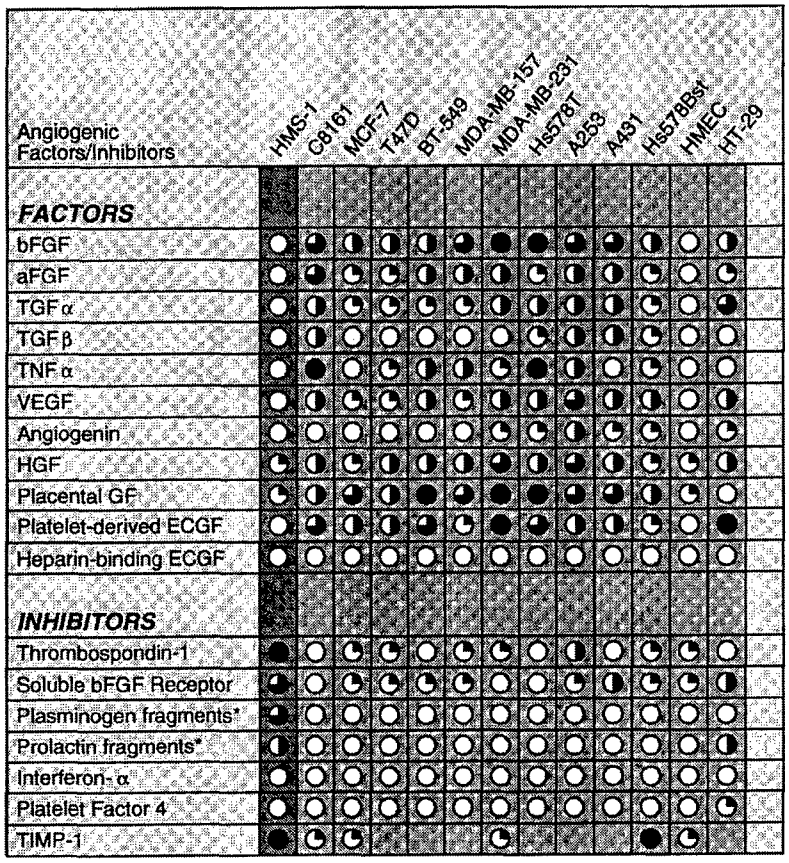
HMEC further supported our contention that HMS-1, though immortal, expressed a well-differentiated myoepithelial phenotype. In addition, because HMS-1 was a clonal line expressing a pure myoepithelial phenotype, it would be predicted to express certain myoepithelial-associated proteins, such as maspin, α1-AT, PNII/APP, and TIMP-1, to a greater degree than HMEC. Predictably, the myofibroblast line Hs578Bst was strongly expressive of TIMP-1 but did not express maspin, PNII, or the 31 kDa inhibitor (Fig. 10.2A). The strong proteinase inhibitor profile exhibited by HMS-1 was shared by all of the myoepithelial xenografts, including HMS-X, HMS-3X, and HMS-4X.

In the modified Matrigel invasion chamber used in this study, HMS-1 cells and their CM dramatically inhibited invasion of two invasive melanoma and breast carcinoma cell lines (Fig. 10.2C, D). The HMS-1 line was itself non-invasive in this chamber. Predictably, the anti-invasive effects of HMS-1 could be abolished by CHX (40 μg/ml) 24-h pretreatment. HMS-1 CM inhibited invasion in a dose-response fashion up to 30% ± 8% of control ($P < 0.01$) (Fig. 10.2D). Pretreatment of HMS-1 with dexamethasone (0.25 μM) produced a complete invasion-permissive phenotype (100% of control), whereas pretreatment with phorbol 12-myristate 13-acetate (PMA; 5 μM) produced an essentially nonpermissive phenotype (2% of control) ($P < 0.05$) (Fig. 10.2E). The effects of dexamethasone and PMA were quite dramatic. The effects of other agents, including RA, dB-cAMP, Na-But, and 5-azaC, showed either permissive or non-permissive trends but were less dramatic. PMA's induction of the nonpermissive phenotype began after 20 min pretreatment, was almost complete after 2 h, and was maximized after 24 h ($P < 0.05$). The induction of this nonpermissive phenotype correlated with the induction of a dramatic fivefold increase in maspin secretion measured in HMS-1 CM. As a result of PMA treatment, both an immediate release (within 2 min) of maspin from HMS-1 cells occurred, as well as a more sustained secretion for at least 24 h following PMA pretreatment. The increased maspin secretion was not on the basis of an increase in steady-state maspin transcripts. PMA also resulted in a less dramatic twofold increase in both MMP-9 and TIMP-1 secretion. Dexamethasone's induction of an invasion-permissive phenotype in HMS-1 was not associated with a change in either maspin transcription or secretion. Immunoprecipitation with anti-maspin antibody at 1/100 dilution successfully removed all detectable maspin from myoepithelial cell CM. This CM lost its ability to inhibit invasion. Similar results were observed with the CM from the other myoepithelial lines (HMS-3 and HMS-4) studied. None of the non-myoepithelial cell CM inhibited invasion.

4. INHIBITION OF TUMOR ANGIOGENESIS

Human myoepithelial cells, which surround ducts and acini of certain organs such as the breast, form a natural border separating epithelial cells from stromal angiogenesis. Myoepithelial cell lines (HMS-1–6), derived from diverse benign myoepithelial tumors, all constitutively express high levels of active angiogenic inhibitors, which include maspin, TIMP-1, thrombospondin-1, and soluble bFGF receptors but very low levels of angiogenic factors [Nguyen et al., 2000] (Fig. 10.3A). Recently, maspin has been shown conclusively to be an angiogenesis inhibitor [Zhang et al., 2000]. As expected, our myoepithelial cell lines inhibited endothelial cell chemotaxis (Fig. 10.3B) and proliferation. These myoepithelial cell lines sense hypoxia and respond to low O_2 tension by increased hypoxia-inducible factor-1α (HIF-1α) but with only a minimal increase in VEGF and nitric oxide synthetase (iNOS) steady-state mRNA levels. Their corresponding xenografts (HMS-X-6X) grow very slowly (Fig. 10.3C) compared with their non-myoepithelial carcinomatous counterparts and accumulate an abundant extracellular matrix devoid of angiogenesis but containing bound angiogenic inhibitors. These myoepithelial xenografts exhibit only minimal hypoxia but extensive necrosis compared with their non-myoepithelial xenograft counterparts. These former xenografts inhibit local and systemic tumor-induced angiogenesis and metastasis, presumably from their matrix-bound and released circulating angiogenic inhibitors. These observations collectively support the hypothesis that the human myoepithelial cell (even when transformed) is a natural suppressor of angiogenesis.

Myoepithelial cells in situ separate epithelial cells from stromal angiogenesis, and this seemingly banal observation serves to illustrate the fact that stromal angiogenesis never penetrates this myoepithelial barrier and raises the hypothesis that myoepithelial cells are natural suppressors of angiogenesis. This observation was reinforced by a microscopic, immunohistochemical, and DNA analysis of our myoepithelial xenografts. Our diverse myoepithelial xenografts secrete and accumulate an abundant extracellular matrix, which is devoid of blood vessels in routine hematoxylin and eosin staining and vWf immunocytochemical staining in contrast to non-myoepithelial xenografts, which show bursts of blood vessels. Microscopic quantitation of vessel density in 10 high power fields reveals absent to low vessel density in the myoepithelial xenografts compared to the non-myoepithelial xenografts ($p < 0.01$) (Fig. 3D). As mentioned previously, murine DNA Cot-1 analysis further reveals the absence of a murine component in the myoepithelial xenografts. Because, in the xenografts, angiogenesis would be murine in origin, the absence of a mur-



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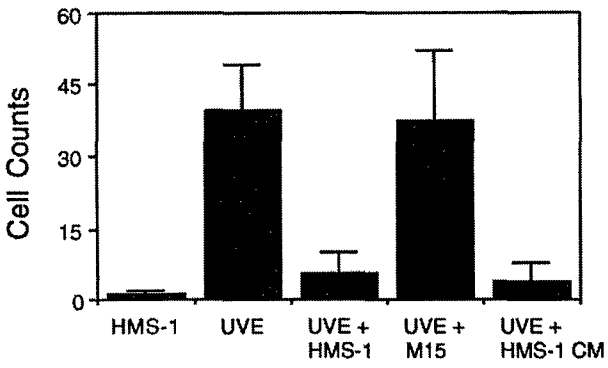
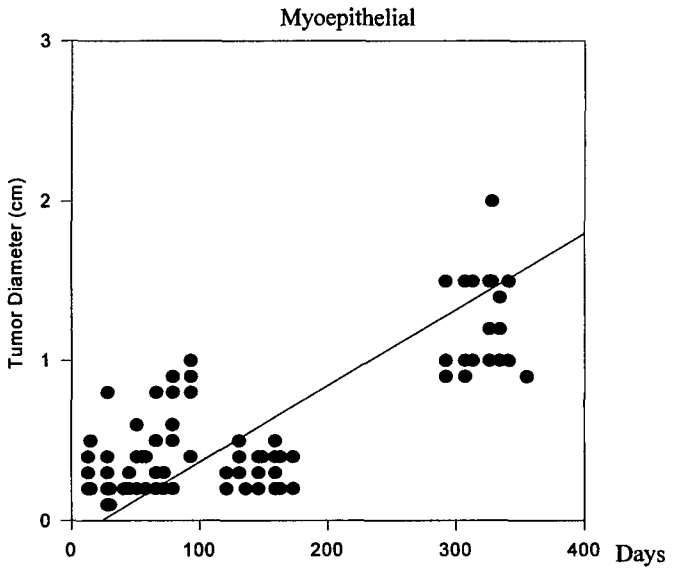
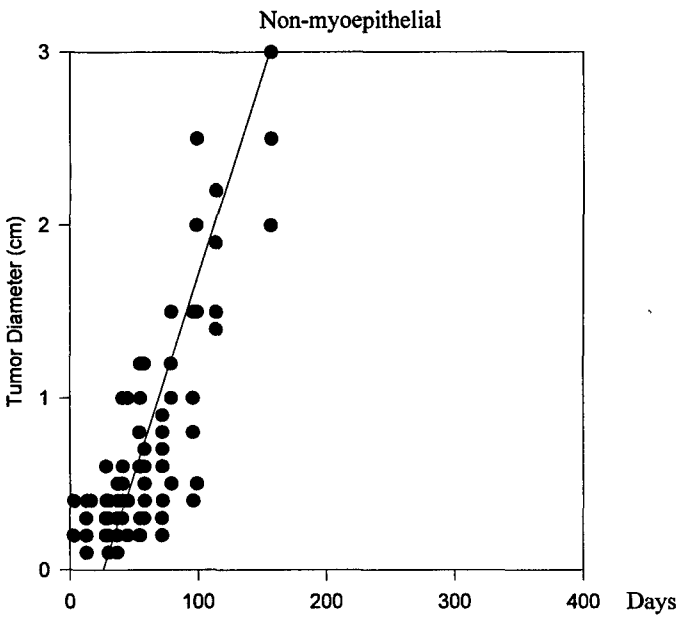


B

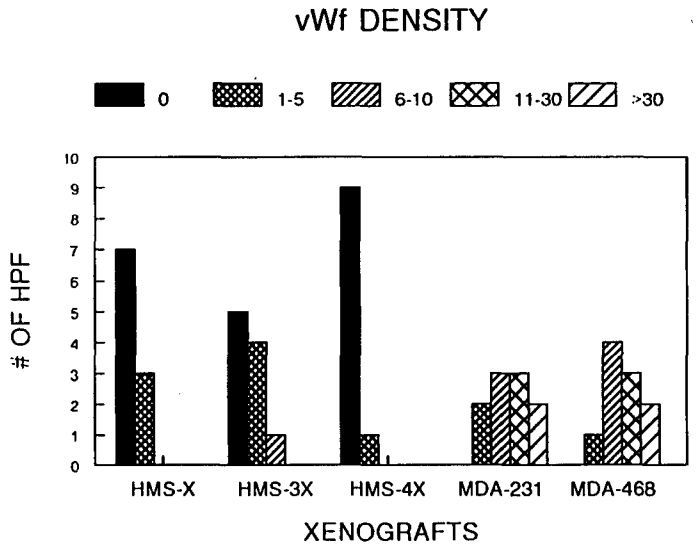
Figure 10.3. A) Relative constitutive gene expression profiles of diverse angiogenic inhibitors and angiogenic factors in HMS-1 compared with numerous other non-myoepithelial cell lines. All measurements were made by Western blot on either CM or HMS-X matrix extracts* and



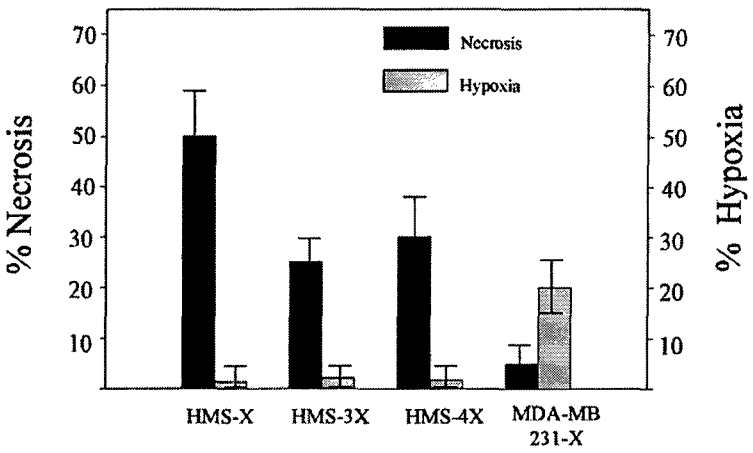


C

Figure 10.3. *(Continued)*
depicted as relative levels of expression. HMS-1 (HMS-X*) uniquely expressed a balance of angiogenic inhibitors over angiogenic factors. B) Inhibition of HUVEC chemotaxis to bFGF by HMS-1 cells and HMS-1 CM is depicted as cell counts collected on the undersurface of a dividing filter. HMS-1 cells themselves were non-migratory. A control non-myoepithelial human melanoma cell line, M15, did not inhibit UVE chemotaxis. C) Growth rates of myo-

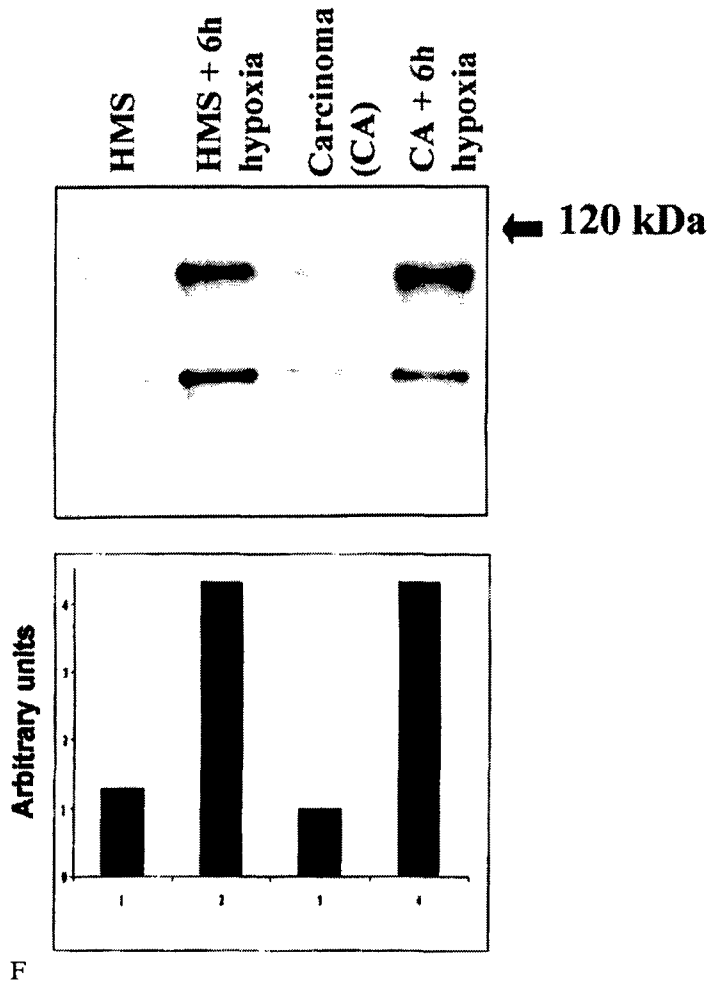


D



E

Figure 10.3. *(Continued)*
epithelial xenografts, for example, HMS-X, compared with growth rates of non-myoepithelial xenografts, for example, MDA-MB-231, revealed comparatively slow myoepithelial growth. This finding suggests a link to endogenously low levels of angiogenesis. D) Density of vWf-positive vessels in 10 H.P.F.'s of myoepithelial versus non-myoepithelial xenografts reveals absent-to-significantly fewer blood vessels in the former xenografts. E) Percentages of hypoxia and percentages necrosis in the myoepithelial versus non-myoepithelial xenografts are contrasted. In the myoepithelial xenografts, necrosis is prominent; whereas, hypoxia is inconspicuous where the reverse is true in the non-myoepithelial xenografts. Under low-O_2 tension, myoepithelial cells; for example, HMS-1 (HMS), like non-myoepithelial carcinoma cells; for example, MDA-MB-231 (CA), show an increase in HIF-1α (F) but, unlike carcinoma cells (CA), show less of an increase in VEGF (G) and iNOS (H) steady-state mRNA levels. Other myoepithelial and carcinoma lines tested exhibited a similar pattern of findings.

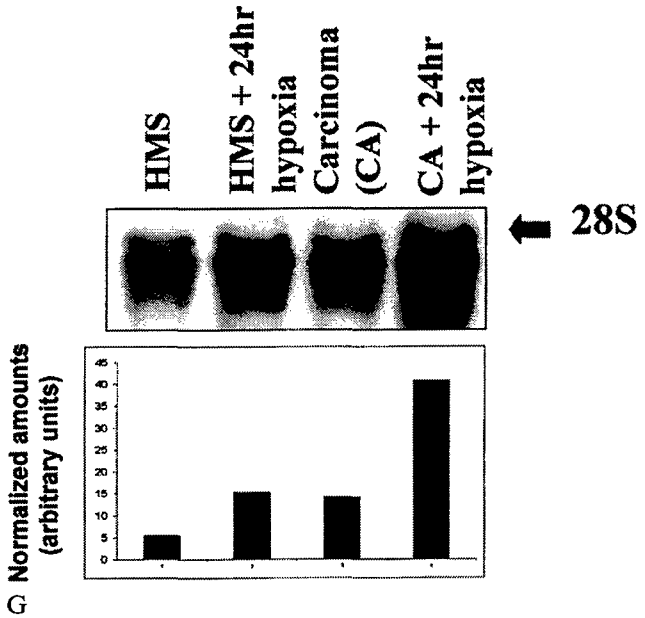


F

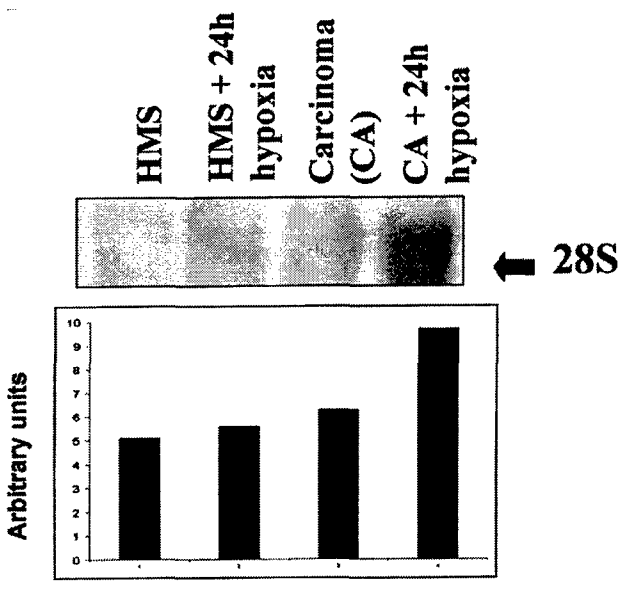
Figure 10.3. *(Continued)*

ine DNA component is another indication that angiogenesis is minimal. As also mentioned, the myoepithelial xenografts grew slowly compared with the non-myoepithelial xenografts, a feature that was not found in comparison between the myoepithelial versus the non-myoepithelial cell lines in vitro.

To explain these in vivo observations, we analyzed the gene expression profiles of our myoepithelial cell lines versus non-myoepithelial cell lines with respect to known angiogenic inhibitors and angiogenic factors. HMS-1, as a prototype myoepithelial cell line, constitutively expressed none of the known angiogenic factors, including bFGF, aFGF, angiogenin, TGF-α, TGF-β, TNF-α, VEGF, PD-ECGF, pla-



G



H

Figure 10.3. *(Continued)*

cenal growth factor (PlGF), IFα, HGF, and HB-EGF, but rather expressed maspin, thrombospondin-1, TIMP-1, and soluble bFGF receptors at high levels; this was in contrast to a high angiogenic factor (which included bFGF, VEGF, TGF-α, TGF-β, HB-EGF, and PD-ECGF) to angiogenic inhibitor gene expression profile, which was observed in non-myoepithelial cell lines.

Other myoepithelial cell lines (HMS-2–6) exhibited an angiogenic inhibitor/angiogenic factor profile similar to that of HMS-1. Interestingly, in 2 M urea extracts of the myoepithelial xenografts, but not in any of the non-myoepithelial xenografts, strong thrombospondin-1, TIMP-1, as well as plasminogen and prolactin fragments could be detected by Western blot. HMS-1 and HMS-1 CM (concentrated 10- to 100-fold) exerted a marked inhibition of endothelial migration (Fig. 10.3B) and proliferation, both of which were abolished by pretreatment of the myoepithelial cells with cycloheximide or dexamethasone. HMS-1 cells themselves did not migrate in response to either K-SFM, FCS, or bFGF.

When mixed with HUVEC, HMS-1 cells reduced endothelial migration to 12% ± 6% of control ($P < 0.01$) (Fig. 10.3B). HMS-1 concentrated CM reduced migration to 8% ± 7% of control ($P < 0.01$). All of the non-myoepithelial malignant human cell lines studied stimulated both endothelial migration and proliferation. Concentrated CM from HMS-1, when fractionated on a heparin-Sepharose column, inhibited endothelial proliferation to 47% ± 10% of control ($P < 0.01$). This inhibitory activity was present only in the 1.5–2.0 M gradient fraction. Pretreatment of HMS-1 cells with PMA resulted in a two- to fivefold increase in endothelial antiproliferative inhibitory activity in both unfractionated CM, as well as in the heparin-Sepharose fraction. Western blot of the heparin-Sepharose column fractions revealed that the 1.5–2.0 M NaCl fraction contained thrombospondin-1. Immunoprecipitation of this fraction with anti-thrombospondin was effective at removing all thrombospondin-1, but it decreased endothelial antiproliferative activity by only 50% and raised the possibility that other angiogenic inhibitors, including maspin, were present in this fraction. The other myoepithelial cell lines (HMS-2–6) exhibited similar antiangiogenic inhibitory activity in their fractionated and unfractionated CM. Therefore, it is likely that both maspin and thrombospondin-1 are anti-angiogenesis effector molecules of myoepithelial cells.

To further explain our in vivo observations of minimal angiogenesis in our myoepithelial xenografts, we performed in vitro and in vivo hypoxia studies. Non-myoepithelial xenografts; for example, MDA-MB-231, exhibited florid hypoxia but only minimal necrosis when they reached a size of 2.0 cm (Fig. 10.3E). In contrast, the myoepithelial xenografts exhibited only minimal hypoxia but prominent ne-

crosis ($P < 0.001$) at the same size of 2.0 cm (Fig. 10.3E). Quantitation of the areas of hypoxia (pimonidazole immunoreactivity) and areas of necrosis in the myoepithelial versus non-myoepithelial xenografts suggested that, in the myoepithelial tumors where angiogenesis is minimal, hypoxic areas progress to necrosis rapidly. In the non-myoepithelial tumors, however, hypoxic areas accumulate but do not progress to necrosis, presumably as a result of the angiogenesis which the hypoxia elicits (Fig. 10.3E).

Comparative analysis of myoepithelial versus non-myoepithelial cell lines to low O_2 tension revealed that, although both cell lines sense hypoxia in that they responded by increasing HIF-1α (Fig. 10.3F), the myoepithelial lines up-regulated their steady-state mRNA levels of the downstream genes, VEGF (Fig. 10.3G) and iNOS (Fig. 10.3H) to a lesser extent than the carcinoma lines, suggesting the possibility of decreased transactivation of hypoxia response element (HRE). Specifically, we observed an approximate 1.7-fold increase in VEGF (1.1-fold increase in iNOS) in myoepithelial cells in response to hypoxia compared to an approximate 2.5-fold increase in VEGF (1.5-fold increase in iNOS) in carcinoma cell lines in response to hypoxia. Although these fold differences by themselves were not impressive, the absolute levels of VEGF (and iNOS) expressed in carcinoma cells in response to hypoxia were 2.5-fold greater for VEGF (and 1.7-fold greater for iNOS) than the levels of VEGF (and iNOS) expressed in myoepithelial cells in response to hypoxia. Therefore, it can be concluded that myoepithelial cells did not express VEGF or iNOS in response to hypoxia to nearly the same extent as carcinoma cells.

To study both local and systemic effects of myoepithelial cells on metastasis, we injected spontaneously metastasizing tumor cells into our myoepithelial xenografts. The highly metastatic *neo*C8161 cells injected into the myoepithelial xenografts could be recovered in significant numbers, although the numbers of clones recovered were less than those recovered from the non-myoepithelial xenografts. Histological analysis of the extirpated xenografts revealed *neo*C8161 cells actively invading through all of the non-myoepithelial xenografts in contrast to the appearance in the myoepithelial xenografts, where the *neo*C8161 cells were confined to the immediate areas around the injection site. Pulmonary metastases of *neo*C8161 were completely absent in the myoepithelial xenograft-injected group, whereas they were quite numerous in the non-myoepithelial group ($P < 0.001$). Analysis of extirpated myoepithelial xenografts containing injected *neo*C8161 cells showed no evidence of murine angiogenesis by either vWf immunocytochemical studies or murine DNA Cot-1 analysis, whereas a similar analysis of extirpated *neo*C8161 injected-non-myoepithelial xenografts showed an increase in murine angiogenesis by

both methods. This suggested that either the matrices of our myoepithelial xenografts or gene product(s) of the myoepithelial cells or both were inhibiting *neo*C8161-induced angiogenesis in vivo. We, in fact, found evidence of maspin, thrombospondin-1, TIMP-1, soluble bFGF receptors, prolactin, and plasminogen fragments within 2 M urea extracts of our myoepithelial xenografts.

In tail-vein injection studies of *neo*C8161 in mice harboring the myoepithelial xenografts, *neo*C8161 formed smaller pulmonary colonies than in mice harboring non-myoepithelial xenografts or in control mice (no xenografts) ($P < 0.01$). In a vWf factor immunocytochemical analysis of these smaller colonies in the mice harboring the myoepithelial xenografts, angiogenesis was minimal. These latter studies suggest the presence of circulating angiogenesis inhibitors released by the myoepithelial xenografts. Just recently we have demonstrated circulating maspin in mice harboring myoepithelial xenografts.

5. PHYSIOLOGICAL AND PHARMACOLOGICAL MANIPULATIONS

Since PMA and dexamethasone were effective at pharmacologically altering maspin levels and the myoepithelial phenotype, we wondered whether physiological agents could do so as well. Because previous basic and clinical studies had examined the role of estrogen agonists and antagonists on human breast cancer cells and because issues of hormone replacement therapy (HRT) and tamoxifen chemoprevention are such timely issues in breast cancer, we wondered whether hormonal manipulations might affect myoepithelial cells in vitro as far as their paracrine suppressive activities on breast cancer were concerned. We recently demonstrated [Shao et al., 2000] that treatment of myoepithelial cells with tamoxifen, but not 17-β estradiol, increases both maspin secretion and invasion-blocking ability. 17-β Estradiol, however, competes with these suppressive effects of tamoxifen, which suggests that the mechanism of tamoxifen action is estrogen-receptor mediated. Myoepithelial cells lack ER-α but express ER-β. Tamoxifen, but not 17-β estradiol, increases AP-1 CAT but not ERE-CAT activity. Again, 17-β estradiol competes with the transcription-activating effects of tamoxifen. These experiments collectively suggest that the actions of tamoxifen on the increased secretion of maspin by myoepithelial cells may be mediated through ER-β and the *trans*-activation of an ER-dependent AP-1 response element.

As mentioned previously, immunoprecipitation of maspin from HMS-1 CM reversed the anti-invasive effects of myoepithelial CM on breast carcinoma cell invasion in vitro. Tamoxifen treatment of HMS-1 resulted in a two- to threefold increase in maspin secretion with

increasing doses of tamoxifen and increasing times of exposure. 17-β Estradiol, in contrast, exerted no effects on maspin secretion and completely abolished the maspin stimulatory effects of tamoxifen in competition experiments. Tamoxifen's increase in maspin secretion was not due to an increase in steady-state maspin mRNA levels, which were essentially unchanged by this treatment. Myoepithelial cell lines lacked ER-α expression but uniformly expressed ER-β. Because estrogen agonists/antagonists bound to estrogen receptors (either ER-α or ER-β) activate downstream genes containing either a classical ERE or an ER-dependent AP-1 response element, myoepithelial cell lines were transfected with CAT-reporter constructs fused to heterologous promoters containing the human estrogen response element (ERE-tk-CAT) or AP-1-tk-CAT. Tamoxifen (10^{-7} M) increased AP-1-CAT activity threefold. This effect was not observed with 17-β estradiol. Furthermore 17-β estradiol (10^{-5} M) competed with and blocked the effects of tamoxifen (10^{-7} M). 17-β estradiol (10^{-7} M) did increase ERE-CAT activity but tamoxifen (10^{-7} M) did not.

6. MYOEPITHELIAL GENE PRODUCTS AS SURROGATE END-POINT MARKERS

Because myoepithelial cells are ubiquitous components of the ductal-lobular units of the breast and other organs, which exhibit branching morphogenesis, we hypothesized that gene products of myoepithelial cells might be detectable in fluid secreted by these ductal-lobular units. As there has been a lot of recent interest in breast ductal fluid and breast nipple aspirates, especially, we measured one myoepithelial gene product, maspin, by Western blot, and found it to be present in both nipple aspirates and ductal fluid but not blood or urine [unpublished observations). These observations indicate that ductal fluid is not a mere transudate of blood or serum and that it is not a product only of epithelial cells (although epithelial protein products, such as casein, lactalbumin, and carcinoembryonic antigen (CEA), are certainly present). Ductal fluid also reflects a significant contribution from myoepithelial cells. From this observation, we are currently studying groups of patients to see whether their maspin levels serve to stratify them. We are currently analyzing ductal fluid collected following cannulation and washing of selected ducts in patients with microcalcifications on screening mammography who are about to undergo either excisional or core biopsy. Paired comparisons of maspin levels in ductal fluid obtained from ducts harboring microcalcifications or DCIS and normal ducts from the same patients are also being made. Maspin levels can be correlated with the histopathology surrounding the microcalcifications. It is anticipated that some of

these patients will exhibit normal ductal histopathology surrounding their microcalcifications, some will harbor proliferations, such as hyperplasia, adenosis, ADH, and DCIS, and still others invasive carcinoma. The screening value of maspin levels in all of these patients can be determined. Measurements of myoepithelial maspin in ductal fluid will be compared with levels of a breast epithelial cell marker, such as CEA. Increased CEA has been observed in nipple secretions and in ductal fluid in patients with ductal hyperplasia. Hence, the maspin/CEA ratio might be predictive of risk with increased maspin/CEA correlating with normalcy and decreased maspin/CEA correlating with high risk, microcalcifications, and/or precancerous histopathology. In this sense, maspin can be used as a surrogate end-point marker to predict either the risk of DCIS or the likelihood that DCIS will progress to invasive breast cancer.

Another interesting observation with respect to the use of myoepithelial maspin as a marker—this time, a tumor marker—is the observation that maspin can be detected in normal saliva but that it is markedly elevated in saliva secreted from a salivary gland neoplasm and that it is also elevated in murine serum in mice harboring human myoepithelial xenografts [unpublished observations]. Most salivary gland neoplasms are thought to be myoepithelial in origin. These include mixed tumors, basal cell adenomas, basal cell adenocarcinomas, and adenoid cystic carcinomas. It was human tumors of these types that originally gave rise to our myoepithelial cell lines/xenografts that led to a dissection of the myoepithelial phenotype and to our observations concerning myoepithelial maspin. If screening saliva for maspin shows promise for detecting small incipient salivary gland neoplasms, then myoepithelial maspin will show its utility as a tumor marker. So, in summary, our findings indicate that the gene products of myoepithelial cells; for example, maspin reflect the structural and functional integrity of the ductal-lobular units of different organs, and alterations in the levels of these myoepithelial gene products in fluid from these units may reflect disease states.

7. METHODS OF CULTURING TRANSFORMED MYOEPITHELIAL CELLS

As has been shown, transformed myoepithelial cells can be derived from benign myoepithelial tumors. The most common site of myoepithelial neoplasia is the salivary glands. Myoepithelial tumors include the pleomorphic adenoma, the basal cell adenoma, the basal cell adenocarcinoma, and the adenoid cystic carcinoma. Although the cells of these tumor are transformed, karyotype analysis is very close to normalcy. Analysis of the gene products of these cells reveals minimal

differences from normal myoepithelial cells; therefore, we believe that these transformed myoepithelial cells can serve as normal myoepithelial cell surrogates. Our laboratory has been successful in establishing six different human myoepithelial cell lines and xenografts using the following methods.

8. PREPARATION OF MEDIA AND REAGENTS

8.1. Supplemented K-SFM

K-SFM keratinocyte serum-free medium supplemented with recombinant epidermal growth factor (EGF, 5 ng/ml) and bovine pituitary extract (BPE, 50 μg/ml)

8.2. Attachment medium

K-SFM with 0.5% FBS.

8.3. CMF-HBSS

Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution.

8.4. Disaggregation medium

F12:DMEM (1:1 mixture of Ham's F12: Dulbecco's modified Eagle's medium) with 10mM HEPES buffer, 2% bovine serum albumin, fraction V, 5 μg/ml Insulin, 300 U/ml Collagenase, and 100 U/ml Hyaluronidase.

8.5. Trypsin-EDTA

Trypsin, 0.05%, 0.7 mM EDTA (disodium ethylene diamine tetraacetate) in CMF-HBSS.

8.6. F12/DMEM/H

F12/DMEM with 15 mM Hepes.

8.7. Dispase medium

F12/DMEM/H with a reduced Ca^{2+} concentration (0.06 mM), and containing 5 U/ml dispase.

8.8. Serum-free F12/DMEM/H

F12:DMEM:H medium supplemented with 1 mg/ml BSA, 1 g/ml insulin, 0.5 g/ml hydrocortisone, 10 ng/ml cholera toxin, 10 ng/ml epidermal growth factor.

8.9. High-salt buffer

3.4 M NaCl, 50 mM Tris-HCl, 20 mM EDTA, 10 mM N-ethylmaleimide (NEM) pH 7.4.

8.10. Urea/guanidinium-HCl extraction buffer

6 M urea, 2 M guanidinium-HCl, 50 mM Tris-HCl, 20 mM EDTA, 10 mM NEM, pH 7.4, with added 2.0 mM dithiothreitol (DTT).

8.11. Tris buffered saline (TBS)

0.15 M NaCl, 50 mM Tris-HCl, 20 mM EDTA, 10 mM NEM, pH 7.4.

Protocol 10.1. Culture of Transformed Human Myoepithelial Cells

Reagents and Materials

Sterile

- Supplemented K-SFM: (see Section 8.1)
- Trypsin-EDTA: (see Section 8.5) Attachment medium: (see Section 8.2)
- CMF-HBSS: (see Section 8.3)
- Dimethylsulfoxide (DMSO)
- Culture dishes, 5 cm
- Culture flasks, 25 cm^2 Scalpels, #11 blade
- Cryovials

Protocol

(a) After human subject consent and approval of the institutional review board, obtain tissues following surgery.

(b) Mince portions of the surgical specimen to ~1 mm^3 under aseptic conditions.

(c) Transfer to dishes containing supplemented K-SFM. Have sufficient medium in the dishes so that the explants are in contact with medium but are able to adhere to the bottom of the dish without being dislodged.

(d) Culture the cells at 37°C in a humidified atmosphere at 5% CO_2 in air.

(e) Change the medium every third day. After 1–2 weeks, cells migrate out from the explants. The medium is suppressive to fibroblast growth but stimulatory to myoepithelial growth.

(f) For subculture of cell monolayers, wash inCMF-HBSS, and detach with trypsin-EDTA.

(g) Resuspend trypsinized cells in attachment medium and allow to attach overnight.

(h) Replace medium with serum-free supplemented K-SFM and maintain thereafter in this serum-free growth medium. Cells are generally seeded in tissue culture flasks at 1/6–1/12 of the confluent density or 0.5–1 × 10^4 cells/cm^2.

(i) Prepare frozen stocks in serum-free medium containing 10% DMSO.

If there is difficulty in establishing the cell line in this manner, you can transplant the initial tumoral explants subcutaneously in the flanks of female nude (nu/nu mutants on a BALB/c background) or SCID mice with a number 10 trochar. We have had a much higher success rate (virtually 100%) with xenograft establishment compared with cell line establishment (10% success rate). The xenografts are very slow growing, however, reaching approximately 1 cm in diameter after 6 months to 1 year (Fig. 10.3C). When the xenografts reach this size, they can be removed by sterile technique, minced, and transplanted to new mice. Portions can also be slow-frozen in serum-free medium containing 10% DMSO and stored in liquid nitrogen. At any point, attempts may again be made to establish a cell line by placing the xenograft explants in cell culture in a manner identical to that for the initial surgical specimen. Using this approach, the success rate for establishing a cell line can be doubled. These strategies are summarized in the Schematic of Figure 10.4.

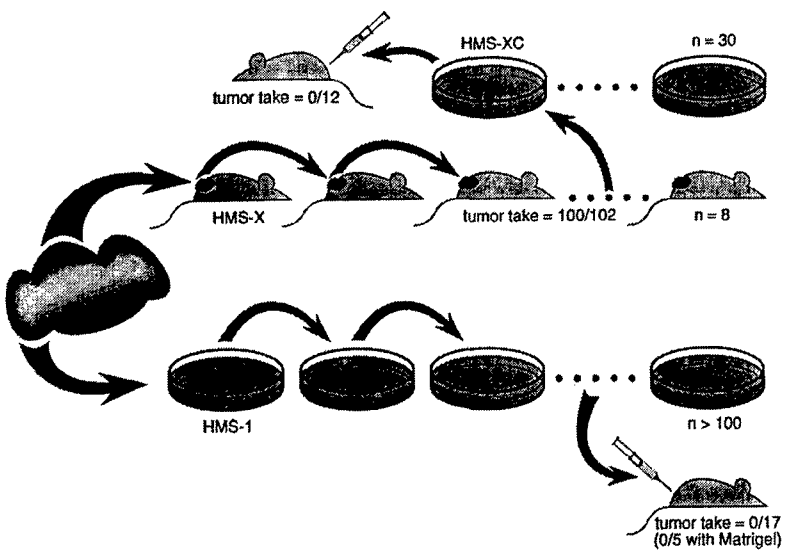


Figure 10.4. Schematic depicts successful method for obtaining myoepithelial cell lines (albeit transformed) from benign myoepithelial tumors of the salivary gland and breast.

9. METHODS OF CULTURING NORMAL MYOEPITHELIAL CELLS

There is no doubt that the use of transformed myoepithelial cells, however convenient, has limitations. For one, these immortalized myoepithelial cells are highly selected and represent homogeneous cell populations, which may not accurately reflect the heterogeneous composition of myoepithelial cells within the normal human mammary gland. Also, transformed myoepithelial cell lines are likely to have acquired genetic changes in long-term culture that confound analysis of the properties of the original myoepithelial cells of the breast or other primary organ. For these reasons, methods to obtain and culture primary normal myoepithelial cells would also be desirable.

Normal breast epithelium consists of different cell types, including luminal epithelial cells that line the ducts and alveoli and basally located myoepithelial cells. These two cell types can be distinguished on the basis of expression of distinct cell markers. The most commonly used phenotypic markers to identify luminal epithelial cells are the MUC-1 apical plasma membrane glycoprotein and cytokeratins 8, 18, and 19. For myoepithelial cells, markers include the common acute lymphocytic leukemia antigen (CALLA/CD10), smooth-muscle actin, cytokeratins 5, 14, and 17, S100, calponin, and maspin. Most studies examining mammary cells in primary culture have utilized the heterogeneous mixed population of epithelial cells (the total epithelial cell population) in the tissue sample. Studies indicate that this heterogeneous mixed population of epithelial cells really consists of luminal epithelial cells and basal myoepithelial cells (see also Chapter 9). Similarly, commercially available HMEC (Clonetics) really consist of both epithelial as well as myoepithelial cells.

Protocol 10.2. Culture of Normal Human Myoepithelial Cells

Reagents and Materials

Sterile

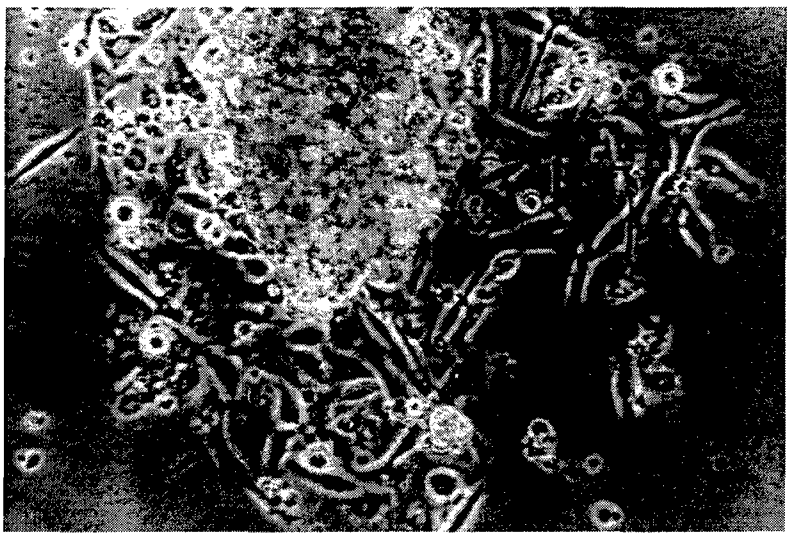
- Disaggregation medium: (see Section 8.4)
- F12/DMEM/H (see Section 8.6) with 5% FCS
- Serum-free F12/DMEM/H (see Section 8.8) or supplemented K-SFM (se Section 8.1)
- Dispase medium: (see Section 8.7)
- Trypsin/EDTA (see Section 8.5)
- DMSO
- Nylon mesh, 20 μm
- Collagen-coated tissue culture dishes or plastic flasks
- Cryovials

Protocol

(a) After human subject consent and approval of an institutional review board, obtain mammary gland tissues (mainly from reduction mammoplasties) following surgery.
(b) After tissue is taken for adequate diagnosis, dissociate the remainder of the tissue and culture the epithelial cells [Emerman et al., 1990; Emerman et al., 1994] as follows.
(c) Trim fat from tissue samples and mince the tissue.
(d) Dissociate by shaking at 37°C for 18 h in disaggregation medium.
(e) Collect the epithelial cell pellet by centrifuging the cell suspension at 80 × g for 4 min.
(f) If a single cell suspension is required; for example, for FACS-sorting, resuspend the cells in trypsin/EDTA for 4 min.
(g) Add F12/DMEM/H containing 5% FCS to inhibit the enzymatic activity of trypsin.
(h) Filter the resultant cell suspension through 20 μm nylon mesh
(i) Centrifuge the filtrate at 100 × g for 5 min, and resuspend the pellet in dispase medium to prevent reaggregation of the cells.
(j) Count the number of viable cells, determined by Trypan blue exclusion, on a hemocytometer.
(k) Seed cells onto collagen-coated tissue culture dishes or plastic flasks.
(l) Culture overnight in F12/DMEM/H containing 5% FCS to allow attachment of cells.
(m) Switch the medium to serum-free F12/DMEM/H or supplemented K-SFM. The purpose of this initial phase of culture is to allow the "total epithelial cells" to adhere and to allow for the selective removal of non-attaching contaminating cells and debris.
(n) At 80% confluence, harvest cells via trypsinization, wash, and freeze in liquid nitrogen as viable cell suspensions.

The average epithelial cell numbers obtained from dissociated normal, dysplastic, and malignant human breast tissue are: normal, 0.5×10^6 cells/g tissue; fibroadenoma, 1.5×10^6 cells/g tissue; fibrocystic, 0.7×10^6 cells/g tissue; carcinoma, 1.0×10^6 cells/g tissue. Cell numbers are increased by culturing. Cell numbers of 5×10^6–1×10^7 can be obtained from primary and early passage cultures, providing sufficient material for experimental manipulations and analyses.

The harvested cells can be analyzed in either two-dimensional or three-dimensional culture systems, both of which reveal two populations of cells: epithelial cells with luminal characteristics and myoepithelial cells with characteristic myoepithelial markers [Yang et al., 1986; Rudland, 1991]. Using either two-dimensional or three-



A

MIXED CELL POPULATION GROWING IN KSFM

↓ ↓

MUC-1 + CALLA- luminal EPITHELIAL CELL

MUC-1-CALLA+ basal MYOEPITHELIAL CELL

↓ ↓

Two dimensional: tightly arranged luminal cells

Two dimensional: teardrop shaped myepithelial cells

↓ ↓

Three dimensional: small spherical alveolar-like colonies

Three dimensional: large branched ductal-like colonies; homogeneous cell types

B

dimensional culture systems, we can detect clonal populations in cultures initiated at low cell densities (<2500 cells/cm^2) with colony yields linearly related to the concentration of cells initially plated. Using either system, we can obtain clonal populations of either cell type at an efficiency of 1–3%. Most colonies generated are composed of 2–10 cells after 14 days, but colonies >50 cells are observed occasionally.

In two-dimensional culture systems; for example, on plastic, the colonies exhibit two major morphologies, which include balls of tightly arranged cells and loosely arranged tear-drop shaped cells having very distinct cell borders. Immunocytochemical analysis of the different colonies has shown that, in the colonies composed of tightly arranged cells, the cells express typical luminal epitopes (MUC-1, keratins 8, 18, and 19) but do not express the myoepithelial marker keratin 14 [Dairkee et al., 1988]. For the colonies composed of the loosely arranged tear-drop shaped cells, the cells express keratin 14, CALLA, smooth-muscle actin, maspin, calponin, S100, and other myoepithelial markers, but do not express the epithelial markers, MUC-1 or keratin 19. These dual populations of cells can be sorted by FACS to yield relatively pure populations of each cell type [O'Hare et al., 1991]. After sorting, the MUC-1$^+$, CALLA$^-$ subpopulation generates further colonies composed of tightly arranged cells. We believe that these are epithelial cells. After sorting, the MUC-1$^-$, CALLA$^+$ subpopulation generates colonies of dispersed teardrop-shaped cells. We think that these cells are myoepithelial cells. Sometimes, in the initial non-sorted population, cells expressing epithelial morphologies and cells expressing myoepithelial morphologies co-exist within a single colony (Fig. 10.5A), with the tightly arranged "luminal" cells in the center of the colony and the teardrop-shaped "myoepithelial" cells radiating out into the periphery of the colony analogous to the in vivo situation.

In three-dimensional culture systems; for example, Vitrogen and *Humatrix*, the mixed population of cells that gives rise to two populations on two-dimensional systems also gives rise to two populations in three-dimensional systems. The mixed population of cells is seeded at low cell density (<1000 cells/ml of gel) within the gel and is

Figure 10.5. (A) Primary cultures of total breast epithelial cells reveal a clump of cuboidal luminal epithelial cells surrounded by more spindly or tear-dropped myoepithelial cells in periphery. These latter cells were CALLA+ and MUC1–. (B) Schematic depicts successful method for obtaining primary myoepithelial cells from human mammary explants and identifying myoepithelial cells out of a mixed cell population based on two- and three-dimensional appearances in tissue culture and confirmatory immunocytochemistry.

cultured in serum-containing medium for 10–14 days. Colonies of varying morphologies emerge with a frequency of ~2%. Colony morphologies include small (<50 cells) spherical colonies composed of a simple cuboidal epithelium surrounding a central lumen, as well as larger (>100 cells) highly branched colonies composed of a solid cord of cells. Immunocytochemical analysis of the colonies generated in three-dimensional systems reveals that the small spherical colonies generally exhibit typical luminal epitopes (MUC-1$^+$, keratins 8/18$^+$, CALLA$^-$, keratin 14$^-$). The larger branched colonies, however, are mainly positive for myoepithelial markers (keratin 14$^+$, CALLA$^+$) and negative for luminal markers (MUC-1$^-$, keratin 19$^-$). Sorting by FACS of the mixed cell populations before seeding within gels (Vitrogen or *Humatrix*) demonstrates that the small spherical colonies derive from the MUC-1$^+$, CALLA$^-$ subpopulation (epithelial cells), and the large branched colonies derive from the MUC-1$^-$, CALLA$^+$ subpopulation (myoepithelial cells). Interestingly, it should be recalled that our transformed myoepithelial cell lines; e.g., HMS-1 give evidence of branching and budding when grown in gels, evidence that they retain this basic myoepithelial characteristic.

The schematic diagram (Fig. 10.5B) summarizes the method for obtaining and identifying myoepithelial cells from human mammary tissues with both two-dimensional and three-dimensional systems.

10. METHODS OF OBTAINING MYOEPITHELIAL MATRIX

Human myoepithelial matrix, termed *Humatrix*, can be obtained from our myoepithelial xenografts [Kedeshian et al., 1998]. This matrix is very rich in proteoglycans and hyaluronic acid (Fig. 10.6A). The method of extraction is as follows.

Protocol 10.3. Preparation of Human Myoepithelial Matrix

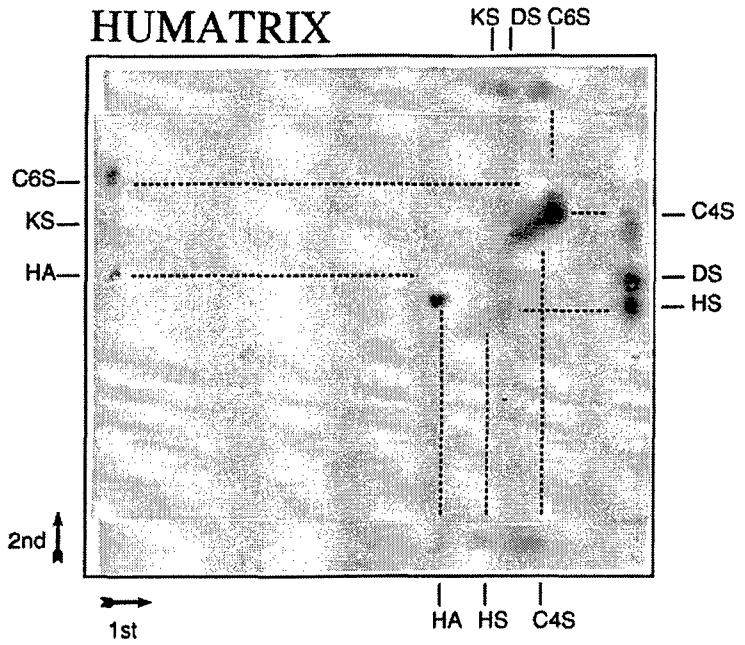
Reagents and Materials

Sterile

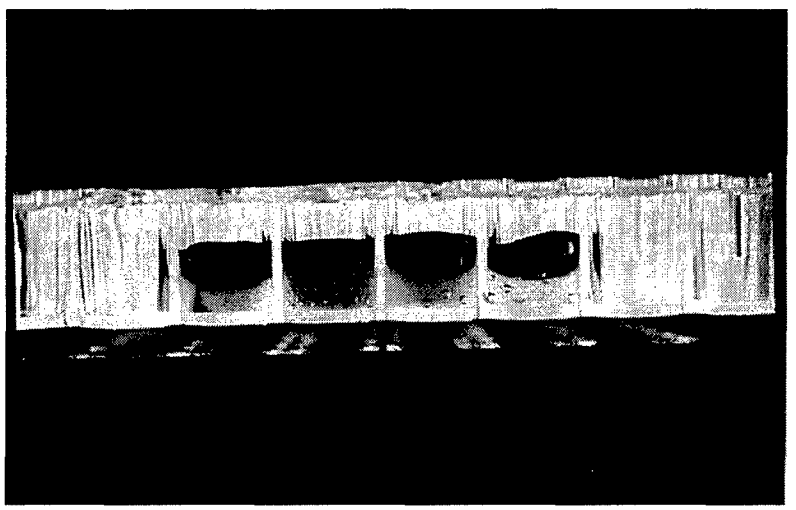
- 0.5% chloroform and cell culture medium

Non-sterile

- β-aminoproprionitrile (BAPN) fumarate fed mice High-salt buffer (see Section 8.9)
- Urea/guanidinium-HCl extraction buffer (see Section 8.10)
- Tris buffered saline (TBS) (see section 8.11)
- Dialysis membrane with a molecular weight cutoff of 5–10 kD

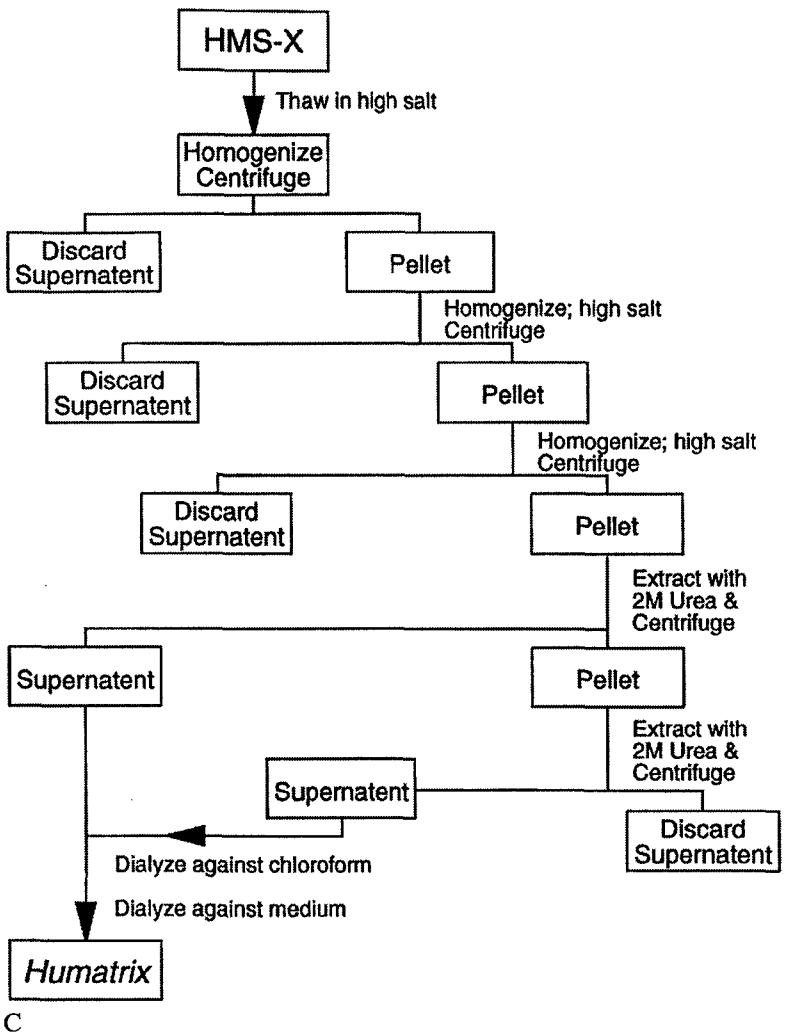


A



B

Figure 10.6. (A) Characteristics of the matrix extract from a human myoepithelial xenograft, HMS-X, is depicted in this 2-D cellulose acetate preparation. Large amounts of chondroitin-4-sulfate proteoglycan, heparan sulfate proteoglycan and hyaluronic acid are evident. (B) This matrix extract undergoes gelation at 25–37°C and excludes cells and large macromolecules. (C) Schematic depicts method of preparation of this human myoepithelial matrix gel, termed *Humatrix*.



C

Figure 10.6. *(Continued)*

Protocol

(a) Grow the tumors in -aminoproprionitrile (BAPN) fumarate fed mice, which are rendered lathyritic.
(b) Harvest HMS-X, HMS-3X, or HMS-4X tumors at 1–2 g of size.
(c) Homogenize 10 g of tumors in 2 ml/g , pH 7.4, at 4°C.
(d) Spin the homogenate for 15 min at 12,000 g at 4°C.

(e) Extract the pellet overnight at 4°C in 0.5 ml urea/guanidinium-HCl extraction buffer per g starting material with gentle stirring.
(f) Spin the extract for 30 min at 24,000 g at 4°C.
(g) Dialyze the supernatant against several changes of TBSat 4°C, followed by sequential dialyses against 0.5% chloroform in cell culture medium by using a dialysis membrane with a molecular weight cutoff of 5–10 kD.
(h) Using the urea/guanidinium-HCl method, 1 ml of *Humatrix* at a protein concentration of 1.5 mg/ml is obtained from each g of HMS-X.
(i) Concentrate the final solution to 3 mg/ml protein by ultrafiltration at 4°C.
(j) Store *Humatrix* at −20°C. At 4°C, *Humatrix* remains liquid; at 25 −37°C *Humatrix* undergoes gelation (Fig. 10.6B).

Humatrix can also be prepared by a pepsin hydrolysis method similar to that used in the preparation of Vitrogen 100. This method of human myoepithelial matrix extraction is summarized in the following schematic (Fig. 10.6C).

11. FUTURE MYOEPITHELIAL RESEARCH DIRECTIONS

The observations that myoepithelial cells secrete suppressive gene products, such as maspin, in large quantities, whereas carcinoma cells do not, suggest that myoepithelial cells exert pleiotropic suppressive effects on tumor progression. Because these gene products are proteinase inhibitors, locomotion inhibitors, and angiogenesis inhibitors, their diverse actions may largely explain the pronounced anti-invasive and anti-angiogenic effects of myoepithelial cells on carcinoma and pre-carcinoma cells. Clearly, the gene products of myoepithelial cells have more than marker value. We need to better understand what it is about the myoepithelial phenotype that allows for high constitutive expression and secretion of tumor suppressive molecules. Studies of the maspin promoter and *cis/trans* interactions within the myoepithelial cell seem to be an attractive line of further research. We also need to understand better the mechanism by which certain pharmacological agents, such as PMA, and certain physiological agents, such as tamoxifen, bolster myoepithelial secretion of suppressive molecules, such as maspin. With this understanding we may be able to design smaller, less-toxic molecules that have the same effect. We need to exploit better the intricate paracrine and local relationships that exist between myoepithelial cells and epithelial cells (precancerous and cancerous) in the breast and other organs. This point is especially im-

portant and timely as intraductal approaches through the nipple are gaining in popularity as a means of screening women who are at risk for developing breast cancer. These intraductal approaches really exploit the local myoepithelial/epithelial relationships that exist. Screening for maspin levels as a surrogate end-point marker is only the beginning. One could envision delivering intraductal gene therapy designed to exploit the inherent differences between myoepithelial and epithelial cells. One could target and destroy the epithelial cells, selectively sparing the myoepithelium or alternately target the myoepithelial cells with a vector, which bolsters its secretion of suppressive molecules. If the myoepithelial defense can be bolstered in this manner, perhaps this natural barrier, which normally inhibits invasion for years, can be made into an impervious barrier, which inhibits invasion forever. At least that is one vision of scientists who are interested in myoepithelial cells.

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SOURCES OF MATERIALS

Item	Supplier
Bovine pituitary extract	Gibco
BSA, fraction V	Gibco
Collagenase	Sigma
Collagen-coated tissue culture dishes or plastic flasks	Collaborative Biomedical Products (BD Biosciences)
Cryovials	Nalge Nunc
Culture dishes and flasks	BD Biosciences
Dispase	Collaborative Biomedical Products (BD Biosciences)
EDTA	Sigma
EGF	Gibco
FCS	Gibco
Ham's F12: Dulbecco's modified Eagle's medium (F12:DMEM)	Stem Cell Technologies Inc.
HEPES	Sigma
Hyaluronidase	Sigma
Insulin	Sigma
K-SFM keratinocyte serum-free medium	Gibco
Nylon mesh	BioDesign
Recombinant EGF	Gibco
Trypsin	Gibco
Vitrogen	Nutacon