Proteomics Demonstration That Normal Breast Epithelial Cells Can Induce Apoptosis of Breast Cancer Cells through Insulin-like Growth Factor-binding Protein-3 and Maspin*

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Normal breast epithelial cells are known to exert an apoptotic effect on breast cancer cells, resulting in a potential paracrine inhibition of breast tumor development. In this study we purified and characterized the apoptosis-inducing factors secreted by normal breast epithelial cells. Conditioned medium was concentrated by ultrafiltration and separated on reverse phase Sep-Pak C_{18} and HPLC. The proapoptotic activity of eluted fractions was tested on MCF-7 breast cancer cells, and nano-LC-nano-ESI-MS/MS allowed the identification of insulin-like growth factor-binding protein-3 (IGFBP-3) and maspin as the proapoptotic factors produced by normal breast epithelial cells. Western blot analysis of conditioned media confirmed the specific secretion of IGFBP-3 and maspin by normal cells but not by breast cancer cells. Immunodepletion of IGFBP-3 and maspin completely abolished the normal cell-induced apoptosis of cancer cells, and recombinant proteins reproduced the effect of normal cell-conditioned medium on apoptosis of breast cancer cells. Together our results indicated that normal breast epithelial cells can induce apoptosis of breast cancer cells through IGFBP-3 and maspin. These findings provide a molecular hypothesis for the long observed inhibitory effect of normal surrounding cells on breast cancer development. ***Molecular & Cellular Proteomics 6: 1239–1247, 2007.***

Breast cancer is the leading cause of cancer-related deaths in women of the western world, and despite significant improvements in cancer diagnosis and treatment, more than two-thirds of the patients still succumb to the disease (1). However, this pathology progresses slowly, and it has been estimated that the development of a clinically detectable tumor from one tumor cell may require 6–8 years. The normal human breast gland comprises a branching ductal-lobular system lined by an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells separated from the interstitial stroma by an intact basement membrane. The luminal epithelial cells are polarized glandular cells with specialized apical and basolateral membrane domains expressing sialomucin and cell-cell adhesion molecules, respectively (2). The myoepithelial cells contribute significantly to the formation of basement membrane, and their myogenic differentiation is responsible for the contractile function. Breast cancer development involves defined clinical and pathological stages starting with atypical epithelial hyperplasia, progressing to *in situ* then invasive carcinomas, and culminating in metastatic disease (3). In *in situ* breast carcinomas, luminal epithelial cells lose their ability to maintain a single epithelial layer. At the same time, the number of myoepithelial cells decreases, and the number of stromal fibroblasts, lymphocytes, and endothelial cells increases. In invasive carcinoma, myoepithelial cells and the basement membrane are absent, and tumor cells are dispersed into the stroma.

It is now widely documented that tumor evolution is highly dependent on interactions between tumor cells and neighboring normal cells (4, 5). The paracrine interactions between neoplastic cells and adjacent normal cells may determine whether transformed cells undergo apoptosis, remain in a quiescent state, or advance to tumorigenesis. In various experimental tumor models, the microenvironment affects the efficiency of tumor formation, the rate of tumor growth, and the extent of invasiveness (6). Although fibroblasts and endothelial cells have been shown to favor tumor development, normal breast myoepithelial and epithelial cells are reported to have antitumor effects both *in vitro* and *in vivo* (7–13). We have demonstrated that normal breast epithelial

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cells (NBECs)[1] inhibit growth of cancer cells by inducing apoptosis (14, 15). The induction of apoptosis is mediated through a Fas-mediated pathway because conditioned medium from NBECs increases membrane-associated Fas and Fas ligand. More importantly both Fas-neutralizing antibody and dominant negative Fas completely abolish NBEC-induced apoptosis. The mechanisms of the apoptosis-inducing effect of NBECs on breast cancer cells, from the standpoint of both effector molecules and signal transduction, hold promise for the understanding of the natural paracrine tumor suppression as well as for cancer prevention. In the present study, we purified and characterized the apoptosis-inducing factors secreted by NBECs. Mass spectrometry analysis together with immunodepletion assay showed insulin-like growth factor-binding protein-3 (IGFBP-3) and maspin to be the major apoptosis-inducing factors produced by NBECs.

EXPERIMENTAL PROCEDURES

Chemicals—All cell culture reagents were obtained from BioWhittaker except insulin, which was obtained from Organon. Chemicals and anti-β-actin antibody were purchased from Sigma unless otherwise stated. Recombinant IGFBP-3 and anti-IGFBP-3 antibodies for Western blot and for immunodepletion assay were obtained from R&D Systems. Anti-maspin antibodies were produced by Biomerieux. Trypsin and soybean trypsin inhibitor were purchased from Roche Applied Science. GST-maspin was produced in *Escherichia coli* as described previously (16).

Cell Culture and Preparation of Conditioned Medium—NBEC cultures were established as described previously (11) from mammoplasty material (18–30-year-old women) obtained from the Department of Plastic Surgery (Prof. Pellerin) at the Medical University of Lille (Lille, France) in accordance with rules and regulations concerning ethical issues in France. In this study, NBECs from three primary cultures were used. Cells were cultured in DMEM/F-12 medium (1:1) containing 5% FCS, 10 μg/ml insulin, 5 μg/ml cortisol, 2 ng/ml EGF, 100 ng/ml cholera toxin, 100 IU/ml streptomycin, 100 μg/ml penicillin, and 45 μg/ml gentamicin. MCF-7, MDA-MB-231 and T-47D breast cancer cell lines were grown in Eagle's minimal essential medium supplemented with 10% FCS, 5 μg/ml insulin, 100 IU/ml streptomycin, 100 μg/ml penicillin, and 45 μg/ml gentamicin. For all experiments, cells were cultured in basal DMEM/F-12 medium without serum.

For preparation of conditioned medium, cells were plated in 75-cm^2 flasks (Nunc). When they reached preconfluence, they were washed two times with PBS and incubated in basal DMEM/F-12 medium (without serum, insulin, cortisol, EGF, or cholera toxin). Two hours later, the basal DMEM/F-12 medium was changed, and cells were further cultured for 24 h. The medium was then centrifuged at 200 $\times$ *g* for 10 min at 4 °C to remove cell debris and stored at −80 °C prior to use.

For immunodepletion of IGFBP-3 and maspin, conditioned medium was incubated with mouse anti-IGFBP-3 and/or anti-maspin antibodies overnight at 4 °C before incubation with protein A-agarose for 2 h on a roller system at 4 °C. Control was performed by incubating conditioned medium with an equal amount of irrelevant mouse immunoglobulin G. After centrifugation (10,000 $\times$ *g* for 10 min at 4 °C), the supernatants were used to determine apoptosis induction.

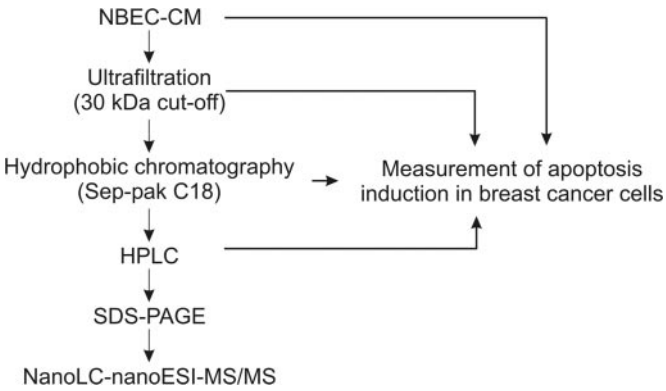


FIG. 1. **Protocol for purification and identification of apoptosis-inducing factors.**

Determination of Apoptotic Cells—Cells were seeded in 35-mm dishes (Nunc). After treatment with NBEC-conditioned medium (NBEC-CM), cells were fixed in cold methanol (−20 °C) for 10 min and washed twice in PBS before staining with 1 μg/ml Hoechst 33258 for 30 min at room temperature in the dark. Cells were then washed with PBS and mounted with coverslips using Glycergel (Dako). Apoptotic cells exhibiting condensed and fragmented nuclei were counted under an Olympus-BH2 fluorescence microscope as described previously (15). At least 500–1,000 cells in randomly selected fields were examined.

Purification Scheme of Apoptosis-inducing Factors—Apoptosis-inducing factors were purified following the protocol described in Fig. 1. To determine the approximate molecular size of apoptosis-inducing factors, size fractionation of NBEC-CM was performed by centrifugation in Centriplus tubes (Millipore) fitted with molecular sieve filters according to the manufacturer's instructions. For further purification, NBEC-CM (10 liters) was concentrated about 1,000 times using an Ultrasette type of ultrafiltration device (Filtron, Pall Gelman Science) (30-kDa cutoff). The concentrate was then loaded onto a reverse phase Sep-Pak C_{18} column and eluted with 50, 80, and 100% acetonitrile. The eluted fractions were freeze-dried and resuspended in DMSO before being loaded onto an HPLC column (Sephasil C_4, 250 $\times$ 10 mm, 5 μm; Vydac). Each eluted fraction was tested for its apoptosis-inducing activity in MCF-7 breast cancer cells as described above.

Protein Identification by Mass Spectrometry—The apoptosis-inducing fractions from HPLC were subjected to 12% SDS-PAGE followed by colloidal Coomassie Blue staining and trypsin digestion. The protein bands of interest were trypsin-digested and analyzed as described previously (17). Nano-LC-nano-ESI-MS/MS analysis of the trypsin digests was performed on an ion trap mass spectrometer (LCQ Deca XP$^+$, Thermo Electron) equipped with a nanoelectrospray ion source coupled with a nano-high pressure liquid chromatography system (LC Packings Dionex). Tryptic digests were resuspended in 10 μl of 0.1% HCOOH, and 1 μl was injected into the mass spectrometer using a Famos autosampler (LC Packings Dionex). The samples were first desalted and then concentrated on a reserve phase precolumn of 5 mm $\times$ 0.3-mm inner diameter (Dionex) by solvent A (H_2O/acetonitrile, 0.1% HCOOH (95:5)) delivered by the Switchos pumping device (LC Packings Dionex) at a flow rate of 10 μl/min for 3 min. Peptides were separated on a 15 cm $\times$ 75-μm-inner diameter C_{18} PepMap column (Dionex). The flow rate was set at 200 nl/min. Peptides were

[1] The abbreviations used are: NBEC, normal breast epithelial cell; Bax, Bcl-2-associated x protein; Bcl-2, B-cell/lymphoma 2; DMEM, Dulbecco's modified minimum essential medium; EGF, epidermal growth factor; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; NBEC-CM, NBEC-conditioned medium; GST-maspin, recombinant GST-maspin fusion protein; uPA, urokinase plasminogen activator.

eluted using a 5–100% linear gradient of solvent B (H_2O/acetonitrile, 0.08% HCOOH (20:80)) in 45 min. Coated nanoelectrospray needles were obtained from New Objective (Woburn, MA). Spray voltage was set at 1.5 kV, and capillary temperature was set at 170 °C. The mass spectrometer was operated in positive ion mode. Data acquisition was performed in a data-dependent mode consisting of, alternatively in a single run, full-scan MS over the range *m/z* 500–2,000 and full MS/MS of the ion selected in an exclusion dynamic mode (the most intense ion is selected and excluded for further selection for a duration of 3 min). MS/MS data were acquired using a 2 *m/z* unit ion isolation window and a 35% relative collision energy. MS/MS raw data files were transformed to dta files with Bioworks 3.1 software (Thermo Electron). MS/MS spectra indicate primarily fragment ions originating from either the C terminus (y ion series) or N terminus (b ion series) of a peptide. Neutral mass of the precursor and sequence information were used to identify proteins in the Swiss-Prot database through MASCOT public interface using a mass tolerance of 0.8 Da for precursor, trypsin as the digestion enzyme, two possible missed cleavages, and oxidized methionine as a variable modification. Results were scored using probability-based Mowse score (protein score is $-10 \times \log(p)$ where p is the probability that the observed match is a random event. Scores greater than 42 are significant ($p < 0.05$). To ascertain unambiguous identification, searches were performed in parallel with Phenyx software using the same parameters.

Western Ligand Blotting and Western Blotting—Conditioned media (2 ml) of NBECs or MCF-7 cells were loaded onto a G-25 Sephadex gel filtration column. Fractions containing IGFBPs were eluted by 0.03 M ammonium acetate and lyophilized as described previously (18). Proteins from lyophilized samples were size-fractionated by 12.5% polyacrylamide gel electrophoresis under non-reducing conditions and electroblotted onto nitrocellulose membranes. The membranes were then incubated overnight in the presence of ^{125}I-labeled IGF-1 and ^{125}I-labeled IGF-2 (2×10^5 cpm for each ligand) (Amersham Biosciences) followed by washing with Tris-buffered saline (pH 7.2) and exposed to Eastman Kodak Co. X-Omat film for at least 48 h. For Western blotting, concentrated media and cell lysates were loaded onto a 10% SDS-polyacrylamide gel and then electrotransferred to nitrocellulose membrane (Hybond-C extra, Amersham Biosciences). After transfer, the blots were blocked with 3% BSA in TBS-T (20 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20) at room temperature and then incubated with anti-IGFBP-3 or anti-maspin antibodies (overnight at 4 °C). The detection was performed using a horseradish peroxidase-conjugated secondary antibody (1.5 h at room temperature) and the ECL detection system (Amersham Biosciences).

Statistical Analysis—Statistical significance was measured by Student's paired *t* test. The value of *p* for each data set is shown in the figures.

RESULTS

NBEC-conditioned Medium Induced Apoptosis of Breast Cancer Cells—MCF-7 breast cancer cells were cultured in serum-free medium in the presence of different dilutions of NBEC-conditioned medium. Apoptosis was determined following Hoechst staining (Fig. 2*A*). As shown in Fig. 2*B*, NBEC-conditioned medium induced apoptosis in a dose-dependent manner with a significant increase in apoptosis at a 1:40 dilution of NBEC-conditioned medium and a 4.5-fold increase in apoptosis at a 1:2 dilution. Similar results in apoptosis induction were obtained using a terminal deoxynucleotidyltransferase biotin-dUTP nick end labeling reaction (data not shown). Conditioned medium was then treated with heat and trypsin to determine whether the apoptosis-inducing factors were proteins. As shown in Fig. 2*C*, heating or trypsin treatment totally suppressed the apoptosis-inducing effect of NBEC-conditioned medium. This indicated that the apoptosis-inducing factors were temperature-sensitive proteins. We then evaluated the approximate molecular masses using a Centricon ultrafiltration system. Conditioned medium was concentrated with membrane filters of different cutoffs (10, 30, 50, and 100 kDa), and the apoptotic activity was determined in non-retained fractions. As shown in Fig. 2*C*, the non-retained fractions from 10- and 30-kDa-cutoff filters could not induce apoptosis, indicating that the molecular masses of apoptosis-inducing factors were greater than 30 kDa. In contrast, the total apoptosis-inducing activity was found in the non-retained fractions when filters of 50- and 100-kDa cutoff were used, indicating that the molecular masses of apoptosis-inducing factors were less than 50 kDa. Therefore the approximate molecular masses of apoptosis-inducing factors were estimated at between 30 and 50 kDa.

Purification of Apoptosis-inducing Factors—NBEC-conditioned medium was sequentially processed as described in Fig. 1. For each step, apoptosis induction of MCF-7 cells was determined. Conditioned medium (10 liters) was concentrated (1000-fold) and then subjected to a reverse phase Sep-Pak C_{18} column and eluted at 50, 80, and 100% acetonitrile. Apoptosis-inducing fractions were subsequently subjected to HPLC. The active fractions a and b were identified from HPLC (Fig. 3*A*). Fraction a was eluted at about 43% acetonitrile. After freeze-drying and dilution to a final concentration equivalent to 50% non-concentrated conditioned medium, this fraction induced about 28% of cancer cells into apoptosis. Fraction b was eluted at about 68% acetonitrile and induced about 17% of cells into apoptosis when cells were treated with a final concentration equivalent to 50% non-concentrated conditioned medium.

Identification of Apoptosis-inducing Factors by Mass Spectrometry—The two apoptosis-inducing fractions eluted by analytical HPLC were subjected to 12% SDS-PAGE followed by colloidal Coomassie Blue staining (Fig. 3*B*). Fraction a was revealed as an apparent double band at about 35–40 kDa. Fraction b was revealed as a single band at 42 kDa. These two individual protein bands were excised from a colloidal Coomassie Blue-stained gel and digested by trypsin, and the resulting peptides were processed for analysis by nano-LC-nano-ESI-MS/MS. The MS/MS spectra and the database search results are presented in Fig. 4. Two peptides were sequenced for each band. The 35-kDa band was identified as IGFBP-3 (score, 156; sequence coverage, 10.6%), and the 42-kDa band corresponded to maspin (score, 300; sequence coverage, 7.8%).

Western Blot Analysis of IGFBP-3 and Maspin—The presence of IGFBP-3 in conditioned media from normal and cancer cells was first verified by Western ligand blot (Fig. 5*A*). In NBEC-conditioned medium, three species of IGFBPs were visualized (IGFBP-2, IGFBP-3, and IGFBP-4) with IGFBP-3

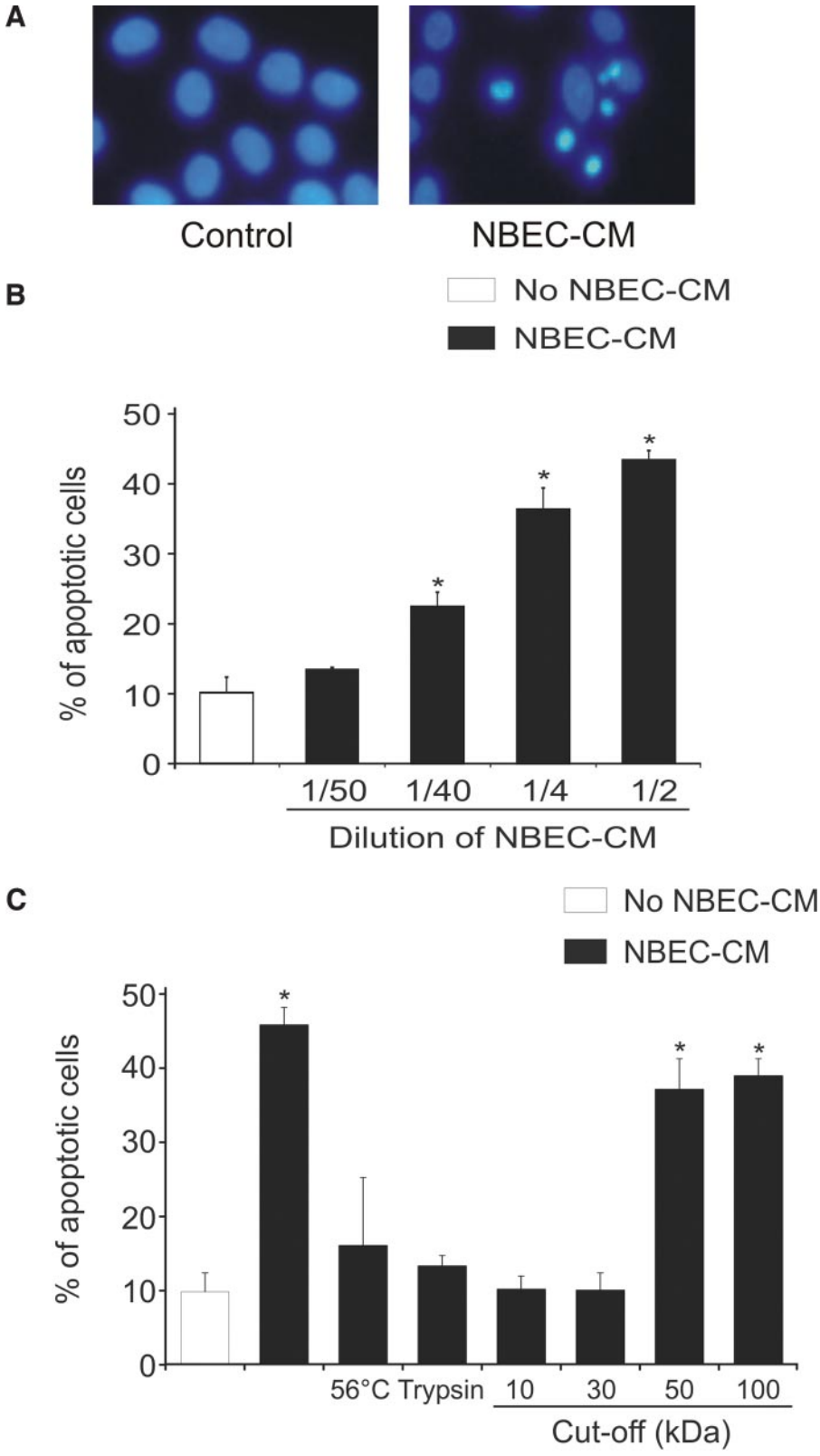


FIG. 2. **NBEC-CM induced apoptosis in MCF-7 breast cancer cells.** Cells were cultured in basal DMEM/F-12 medium for 24 h in the presence or absence of various dilutions of NBEC-CM. *A*, apoptosis detection after Hoechst staining. Apoptotic nuclei are condensed or fragmented. *B*, percentage of apoptotic cells. Data are the mean of three independent experiments. *C*, characterization of apoptosis-inducing activity in NBEC-CM. NBEC-CM was heated at 56 °C for 30 min or incubated with 2.5 μg/ml trypsin at 37 °C for 30 min. Molecular size fractionation of NBEC-CM was performed using 10-, 30-, 50-, and 100-kDa-cutoff sieve filters, and the non-retained fractions were used to determine apoptosis induction in MCF-7 cells as described. Apoptosis experiments were performed with a 1:2 dilution of conditioned medium. *Bars* show S.D. *, $p < 0.01$.

being the most abundant. In medium conditioned by MCF-7 cells, only IGFBP-2 and IGFBP-4 were detected.

The presence of IGFBP-3 and maspin in cell lysates and conditioned media from NBECs and MCF-7 cells was also verified by immunoblot analysis. As shown in Fig. 5, *B* and *C*, both IGFBP-3 and maspin were produced and secreted by

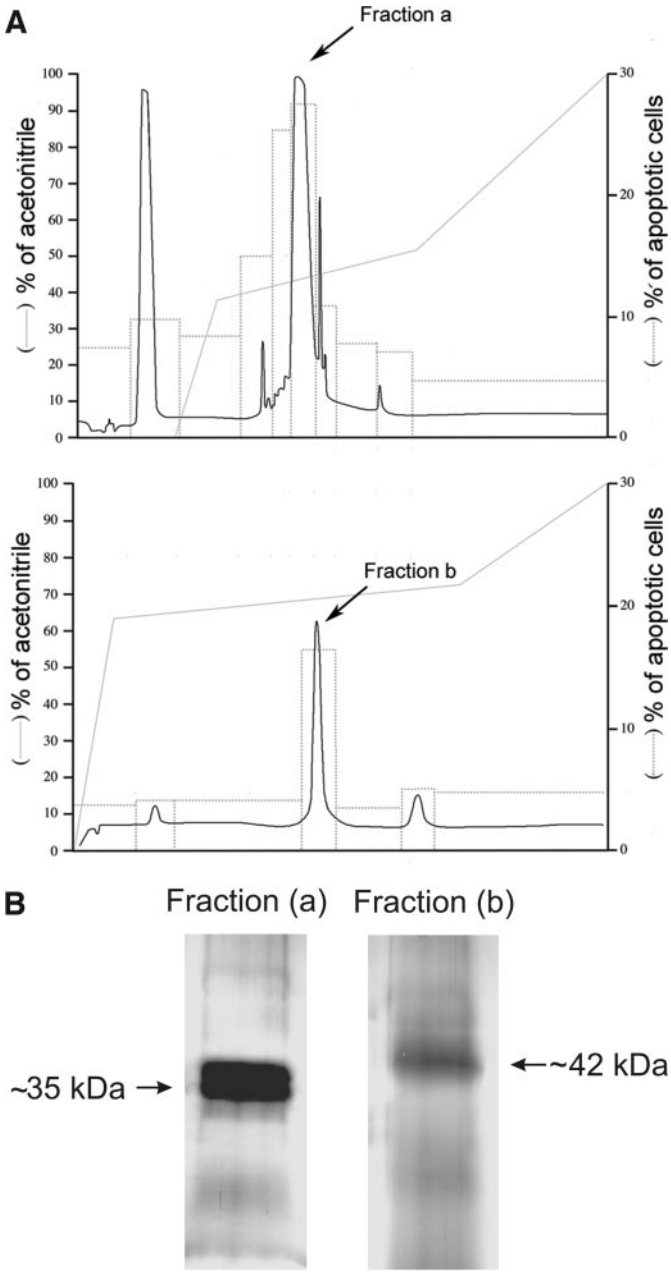


FIG. 3. **Elution profiles of HPLC and SDS-PAGE separation of active fractions.** *A*, HPLC elution profile reveals two active fractions, a and b. HPLC columns were eluted with a linear gradient of 0.1% TFA in acetonitrile. The flow rate was maintained at 1 ml/min, and the absorbance was monitored at 280 nm. Apoptotic activity of collected fractions was determined on MCF-7 cells after Hoechst staining. *B*, SDS-PAGE analysis of HPLC fractions. The active fractions a and b from HPLC were subjected to SDS-PAGE followed by colloidal Coomassie Blue staining.

NBECs. In contrast, neither IGFBP-3 nor maspin was detected in MCF-7 cell lysate and conditioned medium. Altogether these results suggested that IGFBP-3 and maspin were specifically secreted by NBECs but not by MCF-7 breast cancer cells.

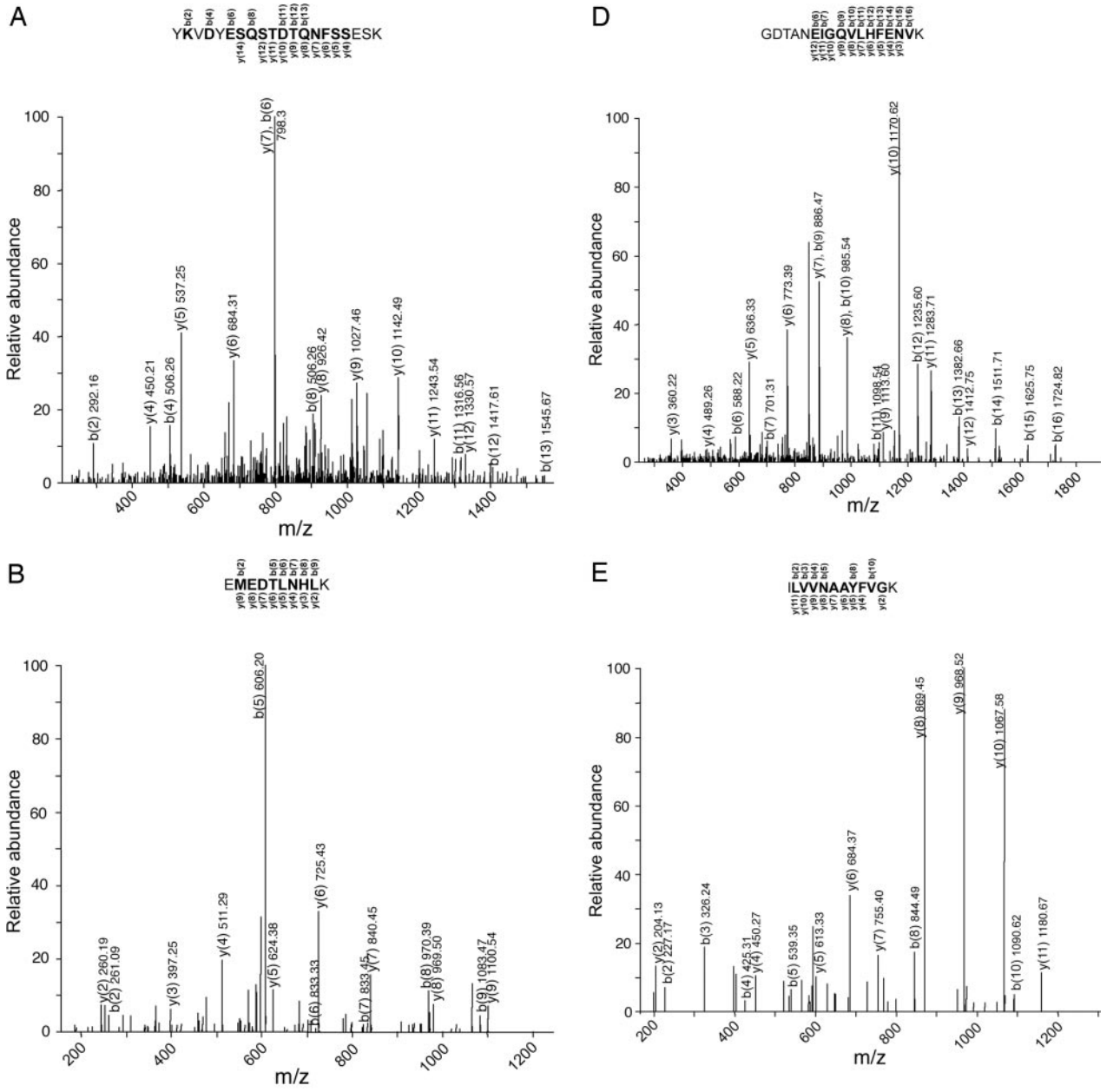
Immunodepletion of IGFBP-3 and Maspin Abolished NBEC-CM-induced Apoptosis—To determine the extent to which secreted IGFBP-3 and maspin contributed to apoptosis induction, we immunodepleted IGFBP-3 and/or maspin by incubating NBEC-CM with anti-IGFBP-3 and/or anti-maspin antibodies. As shown in Fig. 6*A*, immunodepletion of NBEC-CM with the anti-IGFBP-3 antibody or the anti-maspin antibody diminished apoptosis induction in MCF-7 cells. Interestingly immunodepletion of both IGFBP-3 and maspin totally abolished apoptosis induction. This confirmed that IGFBP-3 and maspin secreted by NBECs did induce apoptosis of MCF-7 breast cancer cells.

IGFBP-3 and Maspin Synergistically Induced Apoptosis of Breast Cancer Cells—It has been described that both endogenous overexpressed maspin and IGFBP-3 can induce apoptosis or potentiate apoptosis induction by other agents (16, 19–22). To provide further evidence that extracellular IGFBP-3 and maspin could also induce apoptosis in breast cancer cells, recombinant proteins were used. As shown in Fig. 6*B*, IGFBP-3 and maspin alone significantly induced apoptosis. Interestingly the induction of apoptosis was further increased by cotreatment with IGFBP-3 and maspin.

DISCUSSION

Accumulating evidence suggests that dynamic cell-cell interaction may be as great a determinant of the behavior of a tumor cell as the specific oncogenetic or tumor suppressor alterations occurring within the malignant cells themselves (1–5). Normal breast myoepithelial and epithelial cells have been demonstrated to exert an inhibitory effect on breast cancer cells. We have reported previously that conditioned medium of normal breast epithelial cells strongly induces apoptosis of breast cancer cells (14, 15), but so far the apoptosis-inducing factors have yet to be identified. Here we used a proteomics-based approach to purify and identify apoptosis-inducing factors from normal breast epithelial cells. Proteomics offers the possibility of identifying proteins at very low concentrations, and this is of considerable interest in characterizing paracrine regulators as well as therapeutic targets in various pathologies such as breast cancers (23, 24). The use of sequential chromatography and mass spectrometry as well as immunodepletion assay has allowed us to identify IGFBP-3 and maspin as the two apoptogens secreted by normal breast epithelial cells.

IGFBP-3 is the most abundant of the circulating IGFBPs that bind IGFs with high affinity. IGFBP-3 inhibits cell proliferation and induces apoptosis by its ability to bind IGFs as well as through its IGF-independent effects. Hence IGFBP-3 can induce apoptosis by modulating the expression of Bcl-2 proteins in human breast cancer cells (25). More recently, Lee *et al.* (22) have demonstrated that in response to IGFBP-3 the retinoid X receptor-α-binding partner nuclear receptor Nur77 rapidly undergoes translocation from the nucleus to the mitochondria, resulting in rapid caspase activation. This nuclear



C Insulin-like growth factor binding protein-3
(P 17936)

MQRARPTLWA AALTLLVLLR GPPVARAGAS SGGLGPVVRC
EPCDARALAQ CAPPPAVCAE LVREPGCGCC LTCALSEGQP
CGIYTERCGS GLRCQPSPDE ARPLQALLDG RGLCVNASAV
SRLRAYLLPA PPAPGNASES EEDRSAGSVE SPSVSSTHRV
SDPKFHPLHS KIIIIKKGHA KDSQR**YKVDY** **ESQSTDTQNF**
SSESKRETEY GPCRR**EMEDT** **LNHLK**FLNVL SPRGVHIPNC
DKKGFYKKKQ CRPSKGRKRG FCWCVDKYGQ PLPGYTTKGK
EDVHCYSMQS K

F Maspin
(P 36952)

MDALQLANSA FAVDLFKQLC EKEPLGNVLF SPICLSTSLS
LAQVGAK**GDT** **ANEIGQVLHF** **ENVK**DIPFGF QTVTSDVNKL
SSFYSLKLIK RLYVDKSLNL STEFISSTKR PYAKELETVD
FKDKLEETKG QINNSIKDLT DGHFENILAD NSVNDQTK**IL**
VVNAAYFVGK WMKKFPESET KECPFRLNKT DTKPVQMMNM
EATFCMGNID SINCKIIELP FQNKHLSMFI LLPKDVEDES
TGLEKIEKQL NSESLSQWTN PSTMANAKVK LSIPKFKVEK
MIDPKACLEN LGLKHIFSED TSDFSGMSET KGVALSNVIH
KVCLEITEDG GDSIEVPGAR ILQHKDELNA DHPFIYIIRH
NKTRNIIFFG KFCSP

FIG. 4. **Mass spectrometry identification of IGFBP-3 and maspin.** The SDS-PAGE bands from fractions a and b were subjected to tryptic digestion. The tryptic digests were analyzed by nano-LC-nano-ESI-MS/MS. Spectra of the IGFBP-3 peptides are shown in *A* and *B*, and spectra of maspin are shown in *D* and *E*. The *bold letters* indicate the detected b and y ions matching the predicted ion mass in the database. The detected fragments (*bold letters*) are indicated in the sequence of full-length IGFBP-3 (*C*) and maspin (*F*).

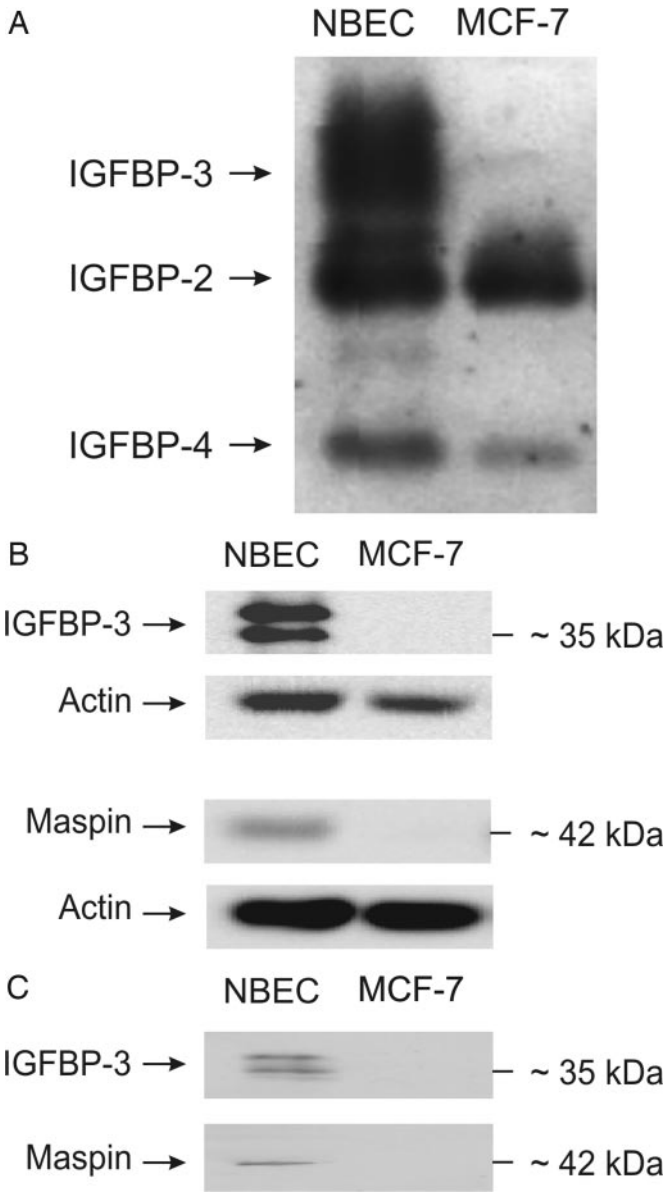


FIG. 5. **Western blot analysis of IGFBP-3 and maspin.** *A*, Western ligand blot analysis of IGFBPs. Conditioned media from NBECs and MCF-7 cells were collected, size-fractionated using 12% SDS-PAGE under non-reducing conditions, electroblotted onto nitrocellulose membranes, and treated with ^{125}I-labeled IGF-1 and -2 as indicated under "Experimental Procedures." *B* and *C*, detection of IGFBP-3 and maspin by Western blotting. Lysates (*B*) and concentrated conditioned media (*C*) from NBECs and MCF-7 cells were subjected to SDS-PAGE and immunoblotted as described under "Experimental Procedures." The loading and transfer of equal amounts of protein were confirmed by immunodetection of actin. The data are representative of three independent experiments.

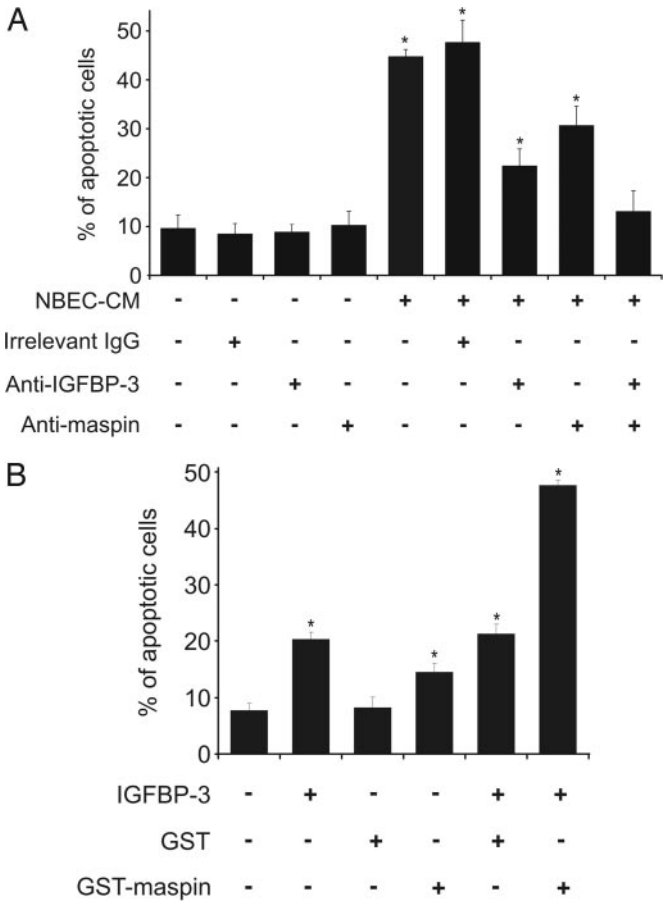


FIG. 6. **Effect of IGFBP-3 and maspin on apoptosis of MCF-7 breast cancer cells.** *A*, effect of IGFBP-3 and maspin immunodepletion from NBEC-conditioned medium. Conditioned medium was incubated with anti-IGFBP-3 and/or anti-maspin antibodies to immunodeplete IGFBP-3 and/or maspin. Irrelevant mouse immunoglobulin G (*IgG*) was used as control. Immunodepleted supernatants were added to MCF-7 cells with a final concentration equivalent to a 1:2 dilution of conditioned medium for 24 h. *B*, effect of recombinant IGFBP-3 and GST-maspin. MCF-7 cells were treated with IGFBP-3 (2.5 ng/ml) or GST-maspin (200 ng/ml) alone or in combination for 24 h. Apoptosis was determined after Hoechst staining. Results are the mean of three independent experiments. Bars show S.D. *, $p < 0.01$.

non-genotypic pathway requires the presence of retinoid X receptor-α. On the other hand, it has also been reported that inducible expression of IGFBP-3 leads to apoptosis induction of MCF-7 breast cancer cells by activating death receptor pathways (21). The level and activity of IGFBP-3 can be controlled by IGFBP-degrading proteases such as metalloproteinases (26, 27). Post-translational modifications including phosphorylation and glycosylation can also modify IGFBP-3 activity. Once phosphorylated, the binding of IGFBP-3 to IGF-I is enhanced (28). Phosphorylation of IGFBP-3 also increases the extracellular translocation of IGFBP-3 into the nucleus to exert IGF-independent effects (29, 30). Glycosylation, which influences bioavailability and cell surface association, may also enhance antiproliferative and proapoptotic effects (31). In our study, IGFBP-3 isolated from NBEC-conditioned medium seemed to be the full-length protein. However, two bands were obtained in the purified HPLC fraction as well as in Western blot analysis. Further studies are required to determine the nature of post-translational modifications of IGFBP-3.

Maspin (mammary serpin) is a serine protease inhibitor with tumor suppression activities. Since its discovery in 1994, maspin has been consistently shown to suppress the aggressive tumor phenotypes, inhibiting cell invasion and mobility *in vitro* and inhibiting tumor growth and metastasis in experimental animal models (32–34). Maspin is localized both in cytoplasmic and nuclear compartments but may also be secreted. Maspin is the only proapoptotic serpin among all the serpins implicated in apoptosis regulation. However, the proapoptotic function of maspin has been so far observed by transfecting cancer cells with maspin cDNA (35). Transfected breast carcinoma cells are more sensitive to multiple apoptotic inducers. This apoptosis-sensitizing effect is mediated through the regulation of Bcl-2 family proteins (20, 35). The intracellular maspin can also translocate to the mitochondria to induce cytochrome *c* release and caspase activation (16). In this work, we showed that both normal breast epithelial cell-secreted maspin and recombinant maspin had the ability to induce apoptosis of breast cancer cells. The underlying mechanism is currently not known; however, maspin has been reported to dramatically decrease both cell-associated uPA/uPA receptor expression and uPA activity (36–38), and it is possible that maspin may induce apoptosis by reducing cell surface-associated prosurvival uPA·uPA receptor complex (34, 39). Alternatively because extracellular maspin is efficiently internalized (36, 40), it is also possible that internalized maspin induces apoptosis in a manner similar to overexpressed intracellular maspin by modulating expression of Bcl-2 family members or by targeting directly mitochondria (16, 20, 35). Consistent with this hypothesis, we have shown previously that conditioned medium from normal breast epithelial cells increases the level of Bax and decreases that of Bcl-2 (41).

Interestingly we showed that the release of IGFBP-3 and maspin is specifically observed for normal breast epithelial cells but not for breast cancer cells. This is consistent with findings that both IGFBP-3 and maspin are transcriptionally down-regulated or silenced by epigenetic changes such as methylation in cancer cells (42, 43). It is important to bear in mind that normal mammary epithelial cells that express high levels of maspin do not undergo detectable apoptosis either *in vitro* or *in vivo* (19). Furthermore maspin expression increases resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)- and staurosporine-induced apoptosis in normal breast epithelial cells (44). Recombinant IGFBP-3 does not modify growth kinetics of normal cells *per se* but can enhance proliferative effects of EGF in normal immortalized breast epithelial cells (MCF-10A) (45). In agreement with these data, we observed that recombinant IGFBP-3 and maspin alone or in combination did not affect growth of normal breast epithelial cells (data not shown). By contrast, MCF-7 breast cancer cells were dramatically induced into apoptosis when cotreated with IGFBP-3 and maspin. In the present study, apoptosis induction in MCF-7 breast cancer cells was used as a functional test to identify apoptosis-inducing factors produced by normal breast epithelial cells. However, we had shown previously that other breast cancer cell lines such as T-47D and MDA-MB-231 cells can also be induced into apoptosis by conditioned medium from normal breast epithelial cells (14, 15). Moreover recombinant IGFBP-3 and maspin synergistically induced apoptosis of these cells (data not shown). These findings indicate that IGFBP-3 and maspin exert a similar effect on distinct breast cell types.

In conclusion, using a proteomics-based approach we identified IGFBP-3 and maspin as the proapoptotic factors produced by normal breast epithelial cells. Although more physiological conditions, such as reconstituted three-dimensional culture systems and animal models, would be required to confirm *in vivo* the role of IGFBP-3 and maspin in breast epithelial cell homeostasis, our data provide a molecular basis for the long observed inhibitory effect of normal surrounding cells on breast cancer development.

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REFERENCES

1. Lacroix, M., Toillon, R. A., and Leclercq, G. (2004) Stable 'portrait' of breast tumors during progression: data from biology, pathology and genetics. *Endocr. Relat. Cancer* **11,** 497–522
2. Ronnov-Jessen, L., Petersen, O. W., and Bissell, M. J. (1996) Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. *Physiol. Rev.* **76,** 69–125
3. Polyak, K. (2001) On the birth of breast cancer. *Biochim. Biophys. Acta* **1552,** 1–13
4. Bissell, M. G., Radisky, D. C., Rizki, A., Weaver, V. M., and Petersen, O. W. (2002) The organizing principle: micro-environmental influences in the normal and malignant breast. *Differentiation* **70,** 537–546
5. Allinen, M., Beroukhim, R., Cai, L., Brennan, C., Lahti-Domenici, J., Huang, H., Porter, D., Hu, M., Chin, L., Richardson, A., Schnitt, S., Sellers, W. R., and Polyak, K. (2004) Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell* **6,** 17–32
6. Elenbaas, B., and Weinberg, R. A. (2001) Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. *Exp. Cell Res.* **264,** 169–184
7. Sternlicht, M. D., Kedeshian, P., Shao, Z. M., Safarians, S., and Barsky, S. H. (1997) The human myoepithelial cell is a natural tumor suppressor. *Clin. Cancer Res.* **3,** 1949–1958
8. Alpaugh, M. L., Lee, M. C., Nguyen, M., Deato, M., Dishakjian, L., and Barsky, S. H. (2000) Myoepithelial-specific CD44 shedding contributes to the anti-invasive and antiangiogenic phenotype of myoepithelial cells. *Exp. Cell Res.* **261,** 150–158
9. Nguyen, M., Lee, M. C., Wang, J. L., Tomlinson, J. S., Shao, Z. M., Alpaugh, M. L., and Barsky, S. H. (2000) The human myoepithelial cell displays a multi-faceted anti-angiogenic phenotype. *Oncogene* **19,** 3449–3459
10. Zajchowski, D. A., Band, V., Trask, D. K., Kling, D., Connolly, J. L., and

Sager, R. (1990) Suppression of tumor-forming ability and related traits in MCF-7 human breast cancer cells by fusion with immortal mammary epithelial cells. *Proc. Natl. Acad. Sci. U. S. A.* **87,** 2314–2318
11. Dong-Le Bourhis, X., Berthois, Y., Millot, G., Degeorges, A., Sylvi, M., Martin, P. M., and Calvo, F. (1997) Effect of stromal and epithelial cells derived from normal and tumorous breast tissue on the proliferation of human breast cancer cell lines in co-culture. *Int. J. Cancer* **71,** 42–48
12. Quarrie, L. H., Pitts, J. D., and Finbow, M. E. (1999) Interactions between normal mammary epithelial cells and mammary tumour cells in a model system. *Cell Prolif.* **32,** 351–361
13. Spink, B. C., Cole, R. W., Katz, B. H., Gierthy, J. F., Bradley, L. M., and Spink, D. C. (2006) Inhibition of MCF-7 breast cancer cell proliferation by MCF-10A breast epithelial cells in coculture. *Cell Biol. Int.* **30,** 227–238
14. Toillon, R. A., Chopin, V., Jouy, N., Fauquette, W., Boilly, B., and Le Bourhis, X. (2002) Normal breast epithelial cells induce p53-dependent apoptosis and p53-independent cell cycle arrest of breast cancer cells. *Breast Cancer Res. Treat.* **71,** 269–280
15. Toillon, R. A., Descamps, S., Adriaenssens, E., Ricort, J. M., Bernard, D., Boilly, B., and Le Bourhis, X. (2002) Normal breast epithelial cells induce apoptosis of breast cancer cells via Fas signaling. *Exp. Cell Res.* **275,** 31–43
16. Latha, K., Zhang, W., Cella, N., Shi, H. Y., and Zhang, M. (2005) Maspin mediates increased tumor cell apoptosis upon induction of the mitochondrial permeability transition. *Mol. Cell. Biol.* **25,** 1737–1748
17. Vandermoere, F., El Yazidi-Belkoura, I., Slomianny, C., Demont, Y., Bidaux, G., Adriaenssens, E., Lemoine, J., and Hondermarck, H. (2006) The valosin-containing protein (VCP) is a target of Akt signaling required for cell survival. *J. Biol. Chem.* **281,** 14307–14313
18. Lalou, C., Lassarre, C., and Binoux, M. (1996) A proteolytic fragment of insulin-like growth factor (IGF) binding protein-3 that fails to bind IGFs inhibits the mitogenic effects of IGF-I and insulin. *Endocrinology* **137,** 3206–3212
19. Jiang, N., Meng, Y., Zhang, S., Mensah-Osman, E., and Sheng, S. (2002) Maspin sensitizes breast carcinoma cells to induced apoptosis. *Oncogene* **21,** 4089–4098
20. Liu, J., Yin, S., Reddy, N., Spencer, C., and Sheng, S. (2004) Bax mediates the apoptosis-sensitizing effect of maspin. *Cancer Res.* **64,** 1703–1711
21. Kim, H. S., Ingermann, A. R., Tsubaki, J., Twigg, S. M., Walker, G. E., and Oh, Y. (2004) Insulin-like growth factor-binding protein 3 induces caspase-dependent apoptosis through a death receptor-mediated pathway in MCF-7 human breast cancer cells. *Cancer Res.* **64,** 2229–2237
22. Lee, K. W., Ma, L., Yan, X., Liu, B., Zhang, X. K., and Cohen, P. (2005) Rapid apoptosis induction by IGFBP-3 involves an insulin-like growth factor-independent nucleomitochondrial translocation of RXRα/Nur77. *J. Biol. Chem.* **280,** 16942–16948
23. Hondermarck, H. (2003) Breast cancer: when proteomics challenges biological complexity. *Mol. Cell. Proteomics* **2,** 281–291
24. Hondermarck, H. (ed) (2004) *Biomedical and Pharmaceutical Applications of Proteomics*, Kluwer-Springer, Dordrecht, The Netherlands
25. Butt, A. J., Firth, S. M., King, M. A., and Baxter, R. C. (2000) Insulin-like growth factor-binding protein-3 modulates expression of Bax and Bcl-2 and potentiates p53-independent radiation-induced apoptosis in human breast cancer cells. *J. Biol. Chem.* **275,** 39174–39181
26. Bernard, L., Babajko, S., Binoux, N., and Ricort, J. M. (2002) The amino-terminal region of insulin-like growth factor binding protein-3, (1–95) IGFBP-3, induces apoptosis of MCF-7 breast carcinoma cells. *Biochem. Biophys. Res. Commun.* **293,** 55–60
27. Miyamoto, S., Yano, K., Sugimoto, S., Ishii, G., Hasebe, T., Endoh, Y., Kodama, K., Goya, M., Chiba, T., and Ochiai, A. (2004) Matrix metalloproteinase-7 facilitates insulin-like growth factor bioavailability through its proteinase activity on insulin-like growth factor binding protein 3. *Cancer Res.* **64,** 665–671
28. Mishra, S., and Murphy, L. J. (2003) Phosphorylation of insulin-like growth factor (IGF) binding protein-3 by breast cancer cell membranes enhances IGF-I binding. *Endocrinology* **144,** 4042–4050
29. Schedlich, L. J., Young, T. F., Firth, S. M., and Baxter, R. C. (1998) Insulin-like growth factor-binding protein (IGFBP)-3 and IGFBP-5 share a common nuclear transport pathway in T47D human breast carcinoma cells. *J. Biol. Chem.* **273,** 18347–18352
30. Schedlich, L. J., Nilsen, T., John, A. P., Jans, D. A., and Baxter, R. C. (2003) Phosphorylation of insulin-like growth factor binding protein-3 by deoxyribonucleic acid-dependent protein kinase reduces ligand binding and enhances nuclear accumulation. *Endocrinology* **144,** 1984–1994
31. Firth, S. M., and Baxter, R. C. (1999) Characterisation of glycosylation variants of insulin-like growth factor binding protein-3. *J. Endocrinol.* **160,** 379–387
32. Shi, H. Y., Zhang, W., Liang, R., Kittrell, F., Templeton, N. S., Medina, D., and Zhang, M. (2003) Modeling human breast cancer metastasis in mice: maspin as a paradigm. *Histol. Histopathol.* **18,** 201–206
33. Bailey, C. M., Khalkhali-Ellis, Z., Seftor, E., and Hendrix, M. J. C. (2006) Biological functions of maspin. *J. Cell. Physiol.* **209,** 617–624
34. Sheng, S. (2006) A role of novel serpin maspin in tumor progression: the divergence revealed through efforts to converge. *J. Cell. Physiol.* **209,** 631–635
35. Zhang, W., Shi, H. Y., and Zhang, M. (2005) Maspin overexpression modulates tumor cell apoptosis through the regulation of Bcl-2 family proteins. *BMC Cancer* **5,** 50
36. Biliran, H. Jr., and Sheng, S. (2001) Pleiotropic inhibition of pericellular urokinase-type plasminogen activator system by endogenous tumor suppressive maspin. *Cancer Res.* **61,** 8676–8682
37. Amir, S., Margaryan, N. V., Odero-Marah, V., Khalkhali-Ellis, Z., and Hendrix, M. J. C. (2005) Maspin regulates hypoxia-mediated stimulation of uPA/uPAR complex in invasive breast cancer cells. *Cancer Biol. Ther.* **4,** 400–406
38. Yin, S., Lockett, J., Meng, Y., Biliran, H. J., Blouse, G. E., Li, X., Reddy, N., Zhao, Z., Lin, X., Anagli, J., Cher, M. L., and Sheng, S. (2006) Maspin retards cell detachment via a novel interaction with the urokinase-type plasminogen activator/urokinase-type plasminogen activator receptor system. *Cancer Res.* **66,** 4173–4181
39. Ossowski, L., and Aguirre-Ghiso, J. A. (2000) Urokinase receptor and integrin partnership: coordination of signaling for cell adhesion, migration and growth. *Curr. Opin. Cell Biol.* **12,** 613–620
40. Odero-Marah, V. A., Khalkhali-Ellis, Z., Chunthapong, J., Amir, S., Seftor, R. E., Seftor, E. A., and Hendrix, M. J. C. (2003) Maspin regulates different signaling pathways for motility and adhesion in aggressive breast cancer cells. *Cancer Biol. Ther.* **2,** 398–403
41. Toillon, R. A., Adriaenssens, E., Wouters, D., Lottin, S., Boilly, B., Hondermarck, H., and Le Bourhis, X. (2000) Normal breast epithelial cells induce apoptosis of MCF-7 breast cancer cells through a p53-mediated pathway. *Mol. Cell. Biol. Res. Commun.* **3,** 338–344
42. Hanafusa, T., Shinji, T., Shiraha, H., Nouso, K., Iwasaki, Y., Yumoto, E., Ono, T., and Koide, N. (2005) Functional promoter upstream p53 regulatory sequence of IGFBP3 that is silenced by tumor specific methylation. *BMC Cancer* **5,** 9
43. Futscher, B. W., Oshiro, M. M., Wozniak, R. J., Holtan, N., Hanigan, C. L., Duan, H., and Domann, F. E. (2002) Role for DNA methylation in the control of cell type specific maspin expression. *Nat. Genet.* **31,** 175–179
44. Lockett, J., Yin, S., Li, X., Meng, Y., and Sheng, S. (2006) Tumor suppressive maspin and epithelial homeostasis. *J. Cell. Biochem.* **97,** 651–660
45. Martin, J. L., Weenink, S. M., and Baxter, R. C. (2003) Insulin-like growth factor-binding protein-3 potentiates epidermal growth factor action in MCF-10A mammary epithelial cells. Involvement of p44/42 and p38 mitogen-activated protein kinases. *J. Biol. Chem.* **278,** 2969–2976