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 C18 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 pro-apoptotic 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 recombining 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) 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 cholaon 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 basic DMEM/F-12 medium without serum.

For preparation of conditioned medium, cells were plated in 75-cm² flasks (Nunc). When they reached confluence, 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 × 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 × g for 10 min at 4 °C), the supernatants were used to determine apoptosis induction. 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 C18 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 C4, 250 × 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 tryptic 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 × 0.3-mm inner diameter (Dionex) by solvent A (H2O/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 × 75-μm-inner diameter C18 PepMap column (Dionex). The flow rate was set at 200 nl/min. Peptides were...
eluted using a 5–100% linear gradient of solvent B (H₂O/ 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 dtf 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 125I-labeled IGF-1 and 125I-labeled IGF-2 (2 × 10⁵ 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. 2A). As shown in Fig. 2B, 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 biont-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. 2C, 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. 2C, 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 C₁₈ 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. 3A). 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. 3B). 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. 5A). In NBEC-conditioned medium, three species of IGFBPs were visualized (IGFBP-2, IGFBP-3, and IGFBP-4) with IGFBP-3
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. 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.
NBECs. In contrast, neither IGFBP-3 nor maspin was detected in MCF-7 cell lysate and conditioned medium. Together 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. 6A, 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. 6B, 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 oncogenic 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
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).
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.
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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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