Short Communication

Nitric Oxide Regulation of Maspin Expression in Normal Mammary Epithelial and Breast Cancer Cells

Zhila Khalkhali-Ellis*[†] and Mary J. C. Hendrix*[†]

From the Department of Anatomy and Cell Biology* and the Holden Comprehensive Cancer Center at The University of Iowa,[†] Carver College of Medicine, The University of Iowa, Iowa City, Iowa

In this study, we examined the unique relationship of maspin, a serine protease inhibitor (serpin), that plays a critical role in mammary gland development and is silenced during breast cancer progression, and nitric oxide (NO), a multifaceted water and lipid soluble free radical. The hypothesis tested was that there is a correlation between endothelial nitric oxide synthase and maspin in MCF-7 cells and that NO is capable of regulating maspin expression. An experimental system was developed in which cellular levels of NO in normal human mammary epithelial cells and the breast cancer cell line MCF-7 could be altered using NO modulators. The effect(s) of NO modulators on maspin was measured using reverse transcriptase-polymerase chain reaction and Western blot analysis of subcellular fractions of both cell types. The data revealed that NO induced maspin expression in MCF-7 cells, and the induced maspin resulted in diminished cell motility and invasion, concomitant with an increase in the apoptotic index. This novel finding provides new information regarding the molecular role of maspin in regulating mammary epithelial growth, remodeling, tumor progression, and the metastatic process. More significantly, these findings could have a potential impact on future therapeutic intervention strategies for breast cancer. Targeted delivery of NO within the tumor microenvironment could provide a feasible noninvasive approach for effective treatment. (*Am J Pathol* 2003, 162:1411-1417)

Nitric oxide (NO), a water and lipid soluble free radical, is generated by a family of NO synthases (NOS).¹ To date, three isoenzymes have been described: endothelial (eNOS) and neuronal (nNOS) enzymes that are constitutively expressed, and the inducible form (iNOS, found in epithelia and macrophages) that is regulated by cyto-

kines.² Both iNOS and eNOS are expressed at high levels in normal mammary epithelium; whereas, the expression of eNOS is down-regulated and iNOS is absent in the breast carcinoma cell line MCF-7.^{3,4} However, the role of these enzymes and their product NO in normal breast development and breast cancer is not understood.

Cellular responses to NO depend on the concentration of NO generated; low levels act as signal transducers, whereas high levels induce apoptosis and could be cytotoxic.⁵⁻⁷ A few studies indicate that NO inhibits tumor cell growth and invasion; whereas other studies suggest that the presence of NO in the tumor microenvironment promotes tumor cell invasion and metastasis.^{8,9} These discrepancies have been attributed to the ability of NO to inhibit apoptosis at low levels and promote the apoptotic cascade at high concentrations.¹⁰ These observations indicate critical dual roles for this free radical in cellular function and tumor cell biology, and provided the basic framework for the current investigation.

Maspin, a serine protease inhibitor (serpin) is present at high concentrations in normal mammary epithelial (and myoepithelial) cells, but its expression is down-regulated in primary breast cancer cell lines and lost in aggressive mammary carcinoma lines.¹¹⁻¹³ Transfection of the mammary carcinoma cell line MDA-MB-435 with maspin cDNA significantly inhibited tumor growth and metastatic ability in nude mice,¹⁴ thus indicating a tumor suppressive activity for this protein as well. In addition, treatment of human breast and prostate cancer cells with recombinant maspin reduced cell motility.^{11,13,14} In the light of these observations, we postulated a possible unique link between the NO system and maspin expression in epithelial cells. We have used an experimental model in which NO levels are modulated using NO inducers, scav-

Supported by the National Institutes of Health (grant NIH/CA 75681 to M. J. C. H.), the American Cancer Society (grant IN no. 122V, administered through the Holden Comprehensive Cancer Center at The University of Iowa to Z. K.-E.), and The Marilyn Rozeboom Endowment from The Order of the Eastern Star (to M.J.C.H.).

Accepted for publication February 11, 2003.

Address reprint requests to Zhila Khalkhali-Ellis, Ph.D., Department of Anatomy and Cell Biology, The Roy J. and Lucile A. Carver College of Medicine, Holden Comprehensive Cancer Center at The University of Iowa, BSB 1-100, Iowa City, IA 52242-1109. E-mail: zhila-ellis@uiowa.edu.

engers, or inhibitors of nitric oxide synthase (NOS) in cell cultures. In addition, eNOS and maspin genes were individually transfected into MCF-7 cells to determine whether the expression of one could induce the re-expression of the other gene. The results have provided compelling evidence regarding the regulation of maspin by NO in both normal mammary epithelial and breast cancer cell lines, and introduce a novel pathway for therapeutic exploitation.

Materials and Methods

Cell Culture

Normal human mammary epithelial cells, N1331, were obtained from Biowhittaker, Inc., Wakersville, MD, and maintained in defined mammary epithelial cell basal medium containing 5 mg/L insulin, 10 μ g/L human epidermal growth factor, 0.5 mg/L hydrocortisone, 52 mg/L bovine pituitary extract, and gentamicin. MCF-7 breast cancer cells were maintained in RPMI 1640 containing 10% fetal calf serum and gentamicin. The modulators used in the proposed studies were tested for possible cell toxicity using the trypan blue exclusion method.

Induction Studies

To address the effect of NO on maspin expression, the following experimental strategies were used: 1) NO scavengers were used to remove endogenous NO; 2) NOS was inhibited with commercially available inhibitors; 3) exogenously produced NO was used; and 4) eNOS was overexpressed in MCF-7 cells.

1) We used NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide, potassium salt (PTIO) (Sigma Chemical Co., St. Louis, MO), to demonstrate the effect of endogenous NO on maspin production in normal and breast cancer cells. MCF-7 and N1331 cells were plated at ~70% confluence and treated with or without PTIO (30 μ mol/L) for 4 to 8 hours. Treated and control cells were analyzed for the mRNA and protein status of maspin. For mRNA studies, total RNA was extracted and subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) analysis using maspin-specific primers to determine maspin mRNA levels in treated and untreated cells. For protein analysis, cells were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer and subjected to subcellular fractionation as described below. The protein content of the fractions was quantified using BCA reagent (Pierce, Rockford, IL). Changes in maspin protein level were determined by Western blot analysis of the subcellular fractions.

2) In the second approach, normal and breast cancer cells were treated with NOS inhibitors *N*^G-nitro-L-arginine methyl ester (L-NAME) or aminoguanidine (Sigma Chemical Co.) to determine the effect of endogenous NO on maspin production. The experimental protocol was identical to that described in strategy 1, with the exception that L-NAME, its inactive stereo isomer *N*^G-nitro-D-arginine methyl ester (D-NAME) (at final concentration of 2

mmol/L), and aminoguanidine (1 mmol/L) were used as modulators.

3) To examine the effect of exogenous NO on maspin and NOS levels in normal and breast cancer cells, we used NO donors such as sodium nitroprusside (SNP) and 1-hydroxy-2-oxo-3-(*N*-ethyl-2-aminoethyl)-ethyl-1-triazene (NOC-12). SNP induces low levels of NO for a short time while NOC-12 causes the release of high levels of NO for a prolonged period.¹⁰ Such a system provides the possibility of addressing the effect of low *versus* high levels of exogenous NO on maspin production in normal and breast cancer cells. These compounds were used at a concentration range of 0 to 1 mmol/L and under culture conditions similar to that described in strategy 1.

4) MCF-7 cells were plated at 70% confluence in six-well culture dishes and transfected with 10⁹ pfu/ml adenoviral vector-expressing wild-type eNOS (AdCMVeNOS,¹⁵ the DNA Core Facility at the University of Iowa) and/or the adenoviral control. eNOS transgene expression and changes in maspin level after transfection were examined by immunohistochemical analysis and/or Western blot of the cell lysate.

Preparation of Subcellular Fractions

Control and treated cells were lysed in buffer A (10 mmol/L HEPES buffer, pH 7.9, containing 10 mmol/L NaCl, 1 mmol/L dithiothreitol, 10% glycerol, 15 mmol/L MgCl₂, 0.2 mmol/L ethylenediaminetetraacetic acid, 0.1% Nonidet P-40, protease inhibitor cocktail, and 1 mmol/L phenylmethyl sulfonylfluoride (PMSF), subjected to three cycles of freeze-thaw and centrifuged at 4500 $\times$ *g* for 10 minutes. The supernatant (cytosolic fraction) was removed, the pellet was washed twice with buffer A, resuspended in buffer B (buffer A containing 500 mmol/L NaCl) and left on ice for 30 minutes with occasional vortexing. The mixture was centrifuged at 25,000 $\times$ *g* for 20 minutes to yield the nuclear fraction. The protein content of each fraction was determined using BCA reagent. Equal amounts of cellular protein (cytoplasmic or nuclear) from various experimental treatments were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis using specific antibodies to maspin, eNOS, or iNOS (BD Pharmingen, San Diego, CA). The reaction products were visualized using the enhanced chemiluminescent kit (Perkin Elmer Life Sciences Inc., Boston, MA). To test for equal loading, the blots were stripped and probed with monoclonal antibody to actin.

Immunohistochemistry

Normal and cancer cell lines were plated on glass coverslips and in 24-well culture dishes (50,000/well). They were then treated with or without modulators and fixed in ice-cold methanol, blocked, and treated with primary antibodies against maspin or NOS. Antibody-antigen complex was visualized using fluorochrome-labeled secondary antibody and viewed using a fluorescence microscope. Untreated cells served as control.

Invasion Assay

The effect of maspin induction on MCF-7 cell invasion was determined using the Membrane Invasion Culture System (MICS) chamber and in the presence or absence of antibody to maspin (3 mg/ml). Briefly, 10^5 cells were seeded randomly in the MICS chamber containing a 10- μ m pore polycarbonate filter (Poretics Corp, Livermore, CA) coated with a defined matrix (50 μ g/ml laminin and collagen IV in a 2 mg/ml gelatin/PBS base). After 24 hours the cells that invaded the matrix were collected and counted as described before.¹⁶

RT-PCR

Total RNA (1 μ g) from control and treated cells was reverse-transcribed using an oligo dT primer and superscript reverse transcriptase (Life Technologies, Gaithersburg, MD). The resulting cDNA was amplified by the PCR using gene-specific 3' and 5' primers for eNOS, iNOS, or maspin. The number of cycles was empirically determined to ensure a linear relationship between input cDNA and gene-specific PCR product. The identity of PCR products was confirmed by sequencing the cloned PCR

fragments. The products were resolved by electrophoresis on a 1% agarose gel containing ethidium bromide.

Enzyme-Linked Immunosorbent Assays (ELISAs)

We used commercial ELISA kits to determine both the basal and induced levels of NO (Active Motif, Carlsbad, CA) in the culture supernatants, the apoptotic index (Roche Molecular Biochemicals, Indianapolis, IN) and MMP-9 and MMP-14 (MT1-MMP) activity (Amersham Biosciences Corp., Piscataway, NJ) in different cell lines after NO modulation.

Results

The status of NOS in breast cancer is not well understood. It is reported that normal mammary epithelial cells express high levels of eNOS and iNOS, whereas in breast cancer cells eNOS is down-regulated and iNOS is reportedly absent.²⁻⁴ To evaluate these parameters in our experimental models, first we attempted to determine the NO levels before and after NO modulation and assessed

Figure 1. A: NO levels in normal mammary epithelial (N1331) and MCF-7 cells before and after different treatment regimens. The effect of NO modulators on eNOS gene expression (B) and protein levels in N1331 and MCF-7 cells (C and D), and iNOSII in N1331 cells (E). Cultures of N1331 and MCF-7 cells (70% confluence) were treated with SNP (0.25 and 0.5 mmol/L), NOC-12 (0.2 mmol/L), L-NAME (2 mmol/L), or PTIO (30 μ mol/L) for 6 hours. Conditioned media were collected for ELISA analysis of NO levels and the cells were used to prepare cytosolic fractions and tested for eNOS and iNOSII levels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis. The membranes were then stripped and reprobed with anti-actin antibody to assess for equal loading.

changes (if any) in transcription, translation, cellular distribution of eNOS and iNOSII after the NO production. ELISA analysis of the conditioned media for nitrite/nitrate levels before and after NO induction is depicted in Figure 1A and indicates minimal basal levels of NO in MCF-7 compared to detectable levels in N1331 cells, under control conditions. Both NO scavenger PTIO and NOS inhibitor L-NAME reduced the basal levels of NO, while SNP and NOC-12 elevated the NO basal level approximately twofold to sixfold (Figure 1A). By using RT-PCR and Western blot analysis of cytosolic proteins, eNOS was detected in N1331 and in MCF-7 cell lines at lower levels (Figure 1; B to D). Apart from the effect of L-NAME (and to some extent NOC-12) that down-regulated eNOS in both cell lines, the basal level of eNOS was not significantly altered by other treatment regimens (Figure 1, C and D). Compared to eNOS, iNOS II was present at much lower levels (both mRNA and protein) in N1331 cells, and with the exception of L-NAME, was not affected by other modulators (Figure 1E).

Our studies using RT-PCR and Western blot analysis confirmed an abundant level of maspin in N1331 normal epithelial cells and very minimal level in MCF-7 cells. Maspin was mostly localized to cytosolic compartment with low levels detectable in nuclear fraction of N1331 cells (Figure 2A). Treatment of N1331 cells with NO modulators had a minimal effect on mRNA levels of maspin in these normal epithelial cells (Figure 2B). At the protein level, a slight reduction in cytosolic maspin in response to high concentrations of SNP [SNP2] and NOC-12 was observed with the appearance of anti-maspin reactive fragment (~38); the inactive stereo isomer D-NAME was ineffective. NO scavenger PTIO exerted a minimal induction effect (Figure 2C). In MCF-7 cells, NO inducers caused a concentration-dependent increase in mRNA and protein levels of maspin with the highest induction observed after NOC-12 treatment. L-NAME treatment had minimal inhibitory effect, while the NO scavenger PTIO exerted a small stimulatory effect (Figure 2, D and E). Both SNP and NOC-12 treatment of MCF-7 cells caused the accumulation of induced maspin in the cytosolic fraction (data not shown). In addition, the NO induction of maspin appeared to be time-dependent.

We then attempted to transiently overexpress eNOS in MCF-7 cells and examine its effect on maspin levels. Ad5eNOS-transfected MCF-7 had increased basal NO levels compared to control MCF-7 cells (6.5 ± 1.3 and $2 \pm 0.7 \mu\text{mol/L}$, respectively). Comparison of control MCF-7 cells with Ad5 viral control and Ad5eNOS-transfected cells by immunohistochemistry and Western blot analysis indicated that eNOS overexpression in MCF-7 cells caused a modest increase in maspin levels (Figure 3A). Interestingly, transfection of these cells with the maspin gene (maspin coding sequence ligated into PEGFP-CZ vector; Clontech, Palo Alto, CA) failed to alter basal eNOS levels (Figure 3B).

Based on previous studies demonstrating that maspin plays a significant role in the motility and invasion of cancer cells,^{14,16} we used the MICS assay to examine whether the induced maspin was functional. We observed that NO mediated induced maspin caused a decrease in the invasive

Figure 2. Maspin expression in normal mammary epithelial and MCF-7 breast cancer cells and its regulation by NO modulators. Subcellular distribution of maspin in N1331 cells as determined by Western blot analysis of cytoplasmic and nuclear fractions (**A**); 25 μg of cellular protein (nuclear or cytosolic) was resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transblotted onto polyvinylidene difluoride membrane, and probed with anti-maspin monoclonal antibody. The effect of NO modulators on maspin expression was determined by RT-PCR and by Western blot analysis of cytoplasmic fractions of N1331 cells (**B** and **C**) and MCF-7 cells (**D** and **E**) treated with or without different NO modulators. Cultures of both cells at 70% confluence were treated with SNP (0.25 and 0.5 mmol/L), NOC-12 (0.2 mmol/L), L-NAME (2 mmol/L), or PTIO (30 $\mu\text{mol/L}$) for 6 hours. For RT-PCR analysis, mRNA was extracted and subjected to RT-PCR using maspin-specific primers. Cytosolic fractions were tested for maspin expression by Western blot analysis. **Arrow** indicates the position of 38-kD anti-maspin reactive band in N1331 cells.

ability of MCF-7 cells, which was reverted by the inclusion of anti-maspin antibody in the invasion assay (Figure 3C). We also tested the treated cells and the conditioned media for changes in the level and activity of cell-associated MT1-

Figure 3. The effect of eNOS gene transfection on maspin expression, and maspin gene transfection on eNOS expression in MCF-7 cells is depicted in (A) and (B), respectively. MCF-7 cells were transfected with 10^9 pfu/ml of AD5eNOS, cytosolic extracts were prepared as described in the Materials and Methods section and tested by Western blot for the expression of eNOS and maspin. Effect of induced maspin on MCF-7 cell invasiveness [in the presence or absence of maspin antibody (C)] was tested by MICS assay system as described in the Materials and Methods section. Changes in the apoptotic index of MCF-7 cells (D) after NOC-12 treatment was determined using commercially available ELISA kits according to the manufacturer's instructions.

MMP (MMP-14) and secreted MMP-2 and MMP-9 after the induction of maspin. Collectively, Western blot, ELISA, and zymographic analyses showed no detectable changes in the level and/or activity of the MMPs (data not shown).

By using cell death detection ELISA kits, we observed that high levels of NO (produced by NOC-12) stimulated apoptosis in MCF-7 cells in a concentration-dependent manner (Figure 3C). However, inclusion of maspin antibody during the induction process reduced the apoptotic index by ~5%. High concentrations of SNP [0.5 mmol/L (SNP2)] were also apoptosis inductive, while other NO modulators were not effective (data not shown). Examination of the eNOS-transfected MCF-7 cells indicated that overexpression of eNOS is also associated with some apoptosis in MCF-7 cells (comparable to the level

induced by SNP treatment), while minimal apoptosis was observed in Ad5 control-transfected MCF-7 cells.

Discussion

Our studies provide the first biological link between maspin and the NOS system in human normal mammary epithelial and breast carcinoma cell lines. These findings demonstrate that modulating the endogenous and exogenous NO levels results in differential regulation of maspin in the normal and cancer cell lines used in this study. Furthermore, exogenously produced NO caused the induction of maspin in maspin-deficient MCF-7 cells, while it reduced cytosolic maspin levels in normal mam-

mary epithelial cells. Generation of maspin fragment with the molecular weight of 38 kd suggests the possibility that NO causes removal of the maspin reactive site loop and directs its lysosomal degradation, an intriguing supposition that may further elucidate the unique structure-function aspects of this novel tumor suppressor gene. Our studies also indicate that transfection of the eNOS gene into MCF-7 is associated with a modest induction in maspin protein levels. It is possible that transfection with eNOS alone is not sufficient for full induction of maspin. In addition, the absence of any changes in eNOS levels after re-expression of maspin in MCF-7 cells indicates that maspin is downstream of eNOS. The data generated from the MICS assay indicate that the induced maspin is functional because it reduces the motility and invasiveness of MCF-7 cells. However, it fails to alter the production and activity of the cell-associated MMP-14 and secreted MMP-2 and MMP-9. Additional studies from our laboratory indicate that maspin suppresses breast cancer cell invasiveness by modulating integrin expression and function¹⁷ and altering Rac level and activity (Odero-Marah VA, Khalkhali-Ellis Z, Chunthapong J, Amir S, Seftor REB, Seftor EA, Hendrix MJCH, submitted).

The findings reported here extend previous work regarding the presence of maspin in normal mammary epithelial cells and its absence in MCF-7 cells.^{11–13} The subcellular fractionation and immunohistochemical analyses indicate that maspin is present in both cytoplasmic and nuclear fractions of normal mammary epithelial cells (with the former being much more prominent). This further confirms the reported observations that maspin could locate to the nucleus,^{17,18} and thus, perform a significant and yet unidentified role in epithelial cell function. This premise is supported by the studies of Maass and colleagues,¹⁹ which indicate strong maspin expression in both nuclear and cytoplasmic compartments of invasive ductal adenocarcinoma of the pancreas. Interestingly, normal pancreatic epithelial cells are usually devoid of either nuclear or cytoplasmic maspin. Furthermore, a recent study from our laboratory has demonstrated the unique phosphorylation of maspin, thus suggesting its potential role in signal transduction events.²⁰

The regulatory role of NO in apoptosis has been widely studied, especially its apoptosis-inducing potential that is concentration-dependent, ie, inhibiting apoptosis at low levels and promoting it at high concentrations.²¹ The proapoptotic effects of NO have been attributed to accumulation of the tumor suppressor protein p53, caspase activation, chromatin condensation, and DNA fragmentation.^{22–27} Our current studies have revealed that high concentrations of exogenous NO promote apoptosis in MCF-7 cells while low levels of exogenous NO (ie, SNP released NO) and transfection of eNOS gene do not exert the same effect on MCF-7 cells. The inclusion of an inhibitory maspin antibody during the NOC-12 treatment reduces the apoptotic index by ~5%, thus indicating a contributory role for maspin in apoptosis. Experiments using anti-sense oligonucleotides are underway to further assess the direct effect of maspin on apoptosis. In addition, it appears that NO exerts phenotype-specific effects on normal *versus* cancerous mammary epithelial cells, as

the same concentration of NO exerted minimal apoptotic effects on normal epithelial cells, but induced degradation of cytosolic maspin. These observations provide novel evidence for a previously unidentified role for maspin involving NO-induced apoptosis in MCF-7 cells.

In summary, our studies have illuminated a previously unexplored NO-maspin relationship that can potentiate increased apoptosis in MCF-7 cells, thus revealing a possible new molecular function for maspin in mammary epithelial cells. Identification of maspin in the nuclear fraction of normal mammary epithelial cells lends further credence for a yet unidentified role for this multifaceted gene in cellular function. Such observations warrant further investigation to elucidate the role of nuclear maspin in gene transcription and cellular function, and to decipher the enigmatic down-regulation of maspin and NOS in breast cancer. More importantly, the regulated release of NO within the tumor microenvironment could provide a possible new therapeutic approach to breast cancer treatment.

Acknowledgments

We thank Dr. Donald Heistad, Department of Internal Medicine, and Dr. Beverly Davidson, the DNA Core Facility, University of Iowa, for the generous provision of AdCMVeNOS; and Dr. Naira Margaryan, Department of Anatomy and Cell Biology, University of Iowa, for performing the invasion assay.

References

1. Nathan C: Nitric oxide as a secretory product of mammalian cells. *EMBO J* 1992, 6:3051–3064
2. Reveneau S, Arnould L, Jolimoy G, Hilpert S, Lejeune P, Saint-Giorgio V, Belichard C, Jeannin JF: NOS in human breast cancer is associated with tumor grade, proliferation rate, and expression of progesterone receptor. *Lab Invest* 1999, 79:1215–1225
3. Martin JH, Alalami O, van den Berg HW: Reduced expression of eNOS in a human breast cancer cell line which has acquired estrogen independence. *Cancer Lett* 1999, 144:65–74
4. Zeillinger R, Tantscher E, Schneeberger C, Tschugguel W, Eder S, Sliutz G, Huber JC: Simultaneous expression of nitric oxide synthase and estrogen receptor in human breast cancer cell lines. *Breast Cancer Res Treat* 1996, 40:205–207
5. Schmidt HHHW, Walter U: NO at work. *Cell* 1994, 78:919–925
6. Le WD, Colom LV, Xie WJ, Smith RG, Alexianu M, Appel SH: Cell death induced by beta-amyloid 1–40 in MES 23.5 hybrid clone: the role of nitric oxide and NMDA-gated channel activation leading to apoptosis. *Brain Res* 1995, 686:49–60
7. Nicotera P, Brune P, Bagetta G: Nitric oxide: inducer or suppressor of apoptosis? *Trends Pharmacol Sci* 1997, 18:189–190
8. Dong Z, Staroselsky AH, Qi X, Xie K, Fidler IJ: Inverse correlation between expression of iNOS activity and production of metastasis by K-1735 murine melanoma cell. *Cancer Res* 1994, 54:789–793
9. Duenas-Gonzales A, Isales CM, Abad-Hernandez MD, Gonzales-Sarmiento R, Sanguaza O, Rodriguez-Vommes J: Expression of inducible nitric oxide synthase in breast cancer correlates with metastatic disease. *Mod Pathol* 1997, 10:645–649
10. Mortensen K, Skouv J, Hougaard DM, Larsson LI: Endogenous eNOS modulate apoptosis in cultured breast cancer cells and is transcriptionally regulated by p53. *J Biol Chem* 1999, 274:37679–37684

11. Zou Z, Anisowicz A, Hendrix MJC, Thor A, Neveu M, Sheng S, Rafidi K, Seftor EA, Sager R: Maspin, a serpin with tumor suppressing activity in human mammary epithelial cells. *Science* 1994, 263:526–529
12. Sheng S, Carey J, Seftor EA, Dias L, Hendrix MJC, Sager R: Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. *Proc Natl Acad Sci USA* 1996, 93:11669–11674
13. Sternlicht MD, Kedeshian P, Shao ZM, Safarians S, Barsky SH: The human myoepithelial cell is a natural tumor suppressor. *Clin Cancer Res* 1997, 3:1949–1958
14. Sager R, Sheng S, Pemberton P, Hendrix MJC: Maspin: a tumor suppressing serpin. *Current Topics in Microbiology and Immunology*, 213. Edited by Buntert U and Birchmeier W. Berlin, Springer-Verlag, 1996, pp 51–64
15. Ooboshi H, Chu Y, Rios CD, Faraci FM, Davidson BL, Heistad DD: Altered vascular function after adenovirus-mediated overexpression of endothelial nitric oxide synthase. *Am J Physiol* 1997, 273:265–270
16. Seftor REB, Seftor EA, Sheng S, Pemberton PA, Sager R, Hendrix MJC: Maspin suppresses the invasive phenotype of human breast carcinoma. *Cancer Res* 1998, 58:5681–5685
17. Seftor REB, Otero VA, Seftor EA, Hendrix MJC: Maspin suppresses breast cancer cell invasiveness by modulating integrin expression and function. *Maspin*. Edited by MJCH Hendrix. Georgetown, Landes Bioscience, 2002, pp 84–95
18. Reis-Filho JS, Milanezi F, Schmitt FC: Maspin is expressed in the nuclei of breast myoepithelial cells. *J Pathol* 2002, 197:272–273
19. Maass N, Rolver L, Zeibart M, Nagasaki K, Roudolph P: Maspin locates to the nucleus in certain cell types. *J Pathol* 2002, 197:273–274
20. Maass N, Hojo T, Ueding M, Luttges J, Kloppel G, Jonat W, Nagasaki K: Expression of the tumor suppressor gene Maspin in human pancreatic cancers. *Clin Cancer Res* 2001, 7:812–817
21. Otero V, Khalkhali-Ellis Z, Schneider GB, Seftor EA, Seftor REB, Koland JG, Hendrix MJC: Maspin phosphorylation is involved in the regulation of human breast cancer cell motility. *Biochem Biophys Res Commun* 2002, 295:800–805
22. Kim PK, Zamora R, Petrosko P, Billiar TR: The regulatory role of nitric oxide in apoptosis. *Int Immunopharmacol* 2001, 8:1421–1441
23. Ambs S, Hussain SP, Harris CC: Interactive effects of nitric oxide and the p53 tumor suppressor gene in carcinogenesis and tumor progression. *EMBO J* 1997, 11:443–448
24. Dimmeler S, Zeiher AM: Nitric oxide and apoptosis: another paradigm for the double-edged role of nitric oxide. *Nitric Oxide* 1997, 1:275–281
25. Brüne B, von Knethen A, Sandau KB: Nitric oxide and its role in apoptosis. *Eur J Pharmacol* 1998, 351:261–272
26. Umanskyt V, Ushmorov A, Ratter F, Chlichlia K, Bucur M, Lichtenauer A, Rocha M: Nitric oxide-mediated apoptosis in human breast cancer cells requires changes in mitochondrial functions and is independent of CD95 (APO-1/Fas). *Int J Oncol* 2000, 16:109–117
27. Cheng A, Chan SL, Milhavel O, Wang S, Mattson MP: p38 MAP kinase mediates nitric oxide-induced apoptosis of neural progenitor cells. *J Biol Chem* 2001, 276:43320–43327