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Maspin Regulates Hypoxia-Mediated Stimulation of uPA/uPAR Complex in Invasive Breast Cancer Cells

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Abstract

Maspin, a unique serine proteinase inhibitor (serpin), plays a key role in mammary gland development and is silenced during breast cancer progression. Maspin has been shown to inhibit tumor cell motility and invasion in cell culture, as well as growth and metastasis in animal models. In this study, we investigated the effect of maspin on the regulation of hypoxia-induced expression of urokinase-type plasminogen activator (uPA) and its receptor (uPAR), with respect to invasive potential in metastatic breast cells MDA-MB-231. We hypothesized that maspin can neutralize or mitigate hypoxia- induced expression of uPA/uPAR in metastatic breast cancer cells, resulting in suppression of their invasive potential. To test our hypothesis, we employed the highly invasive MDA-MB-231 breast cancer cells that are devoid of maspin, and transfected them with the *maspin* gene, and then determined the effect of hypoxia on uPA/uPAR expression. Normal mammary epithelial cells 1436N1 were used as a control. Our findings demonstrate that maspin downregulated the basal and hypoxia-induced uPA/uPAR expression and reduced the stimulatory effect of hypoxia on the in vitro invasive ability of MDA-MB-231-cells. In addition, maspin also inhibited the enzymatic activity of secreted and cell associated uPA in MDA-MB-231 cells. These results indicate that maspin inhibits hypoxia-induced invasion of metastatic breast cancer cells by blocking the uPA system, thus illuminating an important molecular pathway for therapeutic consideration.

Keywords

uPA/uPAR; hypoxia; invasion; maspin; breast cancer

INTRODUCTION

The tumor microenvironment is uniquely different from normal tissues. It exhibits abnormal and chaotic vascular networks, unbalanced blood supply, acidic extracellular pH and variations in perfusion.[1] As a consequence, many regions within tumors become chronically hypoxic (0–3% O_2). Studies have indicated that cells exposed to a hypoxic environment exhibit reduced sensitivity to radiation and drug therapy;[2,3] increased ability to invade the extracellular matrix (ECM) in vitro;[4] and greater in vivo metastatic potential.[5] However,

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hypoxia-inducible factor (HIF-1α) which is overexpressed in many cancers including colon, prostate and breast facilitates tumor cells in their interactions with a hostile microenvironment by increasing transcription of many genes.[6,7] The protein products of some of these genes promote angiogenesis (to increase oxygen availability), glycolysis (to decrease oxygen consumption), growth-factor signalling, apoptosis, pH-regulation, invasion and metastasis.[8,9]

In order to invade and metastasize, tumor cells must degrade the ECM, a process facilitated by a combination of proteolytic enzymes, including uPA. UPA is secreted as an enzymatically inactive single chain proenzyme and binds to its cellular receptor uPAR by its amino-terminal fragment (ATF) in an autocrine fashion.[10,11] The binding of uPA to uPAR localizes the enzymatic activity of uPA which can trigger a focal and directional proteolysis of the ECM.[10,12] The complex of uPA-uPAR then catalyses the conversion of plasminogen to plasmin, a broad spectrum enzyme, which leads to degradation of ECM or activation of other zymogens such as the matrix metalloproteinases (MMP's).[13] UPA/uPAR is overexpressed in many cancers, including breast, prostate, colon and lung carcinoma.[14] The increase in uPAR expression has been implicated in the stimulation of invasive potential of tumor cells.[13] For example, transfection of human osteosarcoma cells with uPAR cDNA resulted in a four-fold increase in their invasive potential, compared to the control cells.[15] Other studies have also indicated that exposure of the MDA-MB-231 breast cancer cells to a hypoxic environment increased the expression of uPAR and significantly enhanced the invasive potential of MDA-MB-231.[15]

Since activation of uPA-dependent proteolysis and signaling depends on the binding of uPA to uPAR, inhibition of this interaction has been proposed as a potential therapeutic modality to inhibit tumor progression. Studies using blocking antibodies to uPA/uPAR, high molecular weight inhibitors of uPA-uPAR interaction, and targeting uPA/uPAR gene expression have resulted in reduced tumor growth, invasion and metastasis.[16–19] Furthermore, the activities of uPA and uPAR are also regulated by members of the serine proteinase inhibitor family (SERPINS), including maspin and plasminogen activator inhibitors-1 and 2 (PAI-1, PAI-2).[20]

Maspin, a 42-kDa protein, is present in high concentration in normal mammary epithelial and myoepithelial cells, downregulated in primary breast cancer cell lines and totally lost in invasive breast cancer cells.[21,22] Loss of maspin expression has been correlated with increased malignancy in breast cancer.[22–24] Although experimental studies have demonstrated a tumor suppressive role for maspin at the level of invasion, tumor growth and metastasis,[24–25] there is no information on the role of maspin in the regulation of the uPA system under hypoxic conditions on breast cancer cells.

In this study, we hypothesized that maspin could suppress the hypoxia-induced uPA/uPAR expression of metastatic breast cancer cells. To test this hypothesis, we generated stable maspin expressing transfectants of the highly invasive/metastatic MDA-MB-231 breast cancer cells and tested their levels of uPA/uPAR under hypoxia, compared with their control counterpart. The data reveal that maspin downregulated the basal and hypoxia-induced uPA/uPAR expression and reduced the stimulatory effect of hypoxia on the in vitro invasive ability of the metastatic breast cancer cells. Furthermore, we observed that maspin inhibited the enzymatic activity of both secreted and cell associated uPA. These findings offer new insights into the influence of the tumor suppressor gene maspin on the molecular effects of hypoxia on breast cancer.

MATERIALS AND METHODS

Cells and culture conditions

MDA MB-231, MDA-MB-231 transfected with maspin gene, were maintained and propagated in RPMI-1640 supplemented with 10% fetal calf serum (FCS) and gentamicin sulfate (50 mg/l) (Gemini Bioproducts; Calabasas, CA). Immortalized normal mammary epithelial cells 1436N1 (a gift from Dr. Shijie Sheng, Department of Pathology, Wayne State University School of Medicine, Detroit, MI) was maintained in D complete medium (1:1 α-MEM: Ham's F-12, containing 1% FCS, 1 ng/ml cholera toxin, 10 mM Hepes pH 7.7, 50 μM ascorbic acid and Mito+ serum free supplement). All the cell lines were routinely screened for *Mycoplasma* species (PCR based and rapid detection system, Roche, Indianapolis, IN) and experiments were performed with 80–90% confluent cultures.

Generation of MDA-MB-231-GFP-maspin

Stable maspin-transfectant of breast carcinoma cells MDA-MB-231 were generated using the Lipofectamine (Invitrogen; Carlsbad, CA) protocol as described previously.[26] RT-PCR and Western blot analysis confirmed the expression of maspin in the transfected cells.

Hypoxic culture conditions

For culture under hypoxic conditions, cells were plated on 150-mm culture dishes (Nalgene Nunc International; Rochester, NY) at 80–90% confluency. They were then placed in the airtight hypoxia chamber (Billups-Rothenberg, Modular Incubator Chamber; Del Mar, CA). The chamber was then flushed with a gas mixture containing 1% O_2, 5% CO_2 and 94% N_2 until the oxygen concentration within the chamber reached 1% as measured by a Miniox 1 oxygen analyzer (Catalyst Research Corp, Owings Mills, MD). Under these conditions, the hypoxia chamber equilibrated within 1–2 hours and the O_2 level remained at or below 1% throughout the incubation period (up to 24 hrs).

RNA isolation and semiquantitative RT-PCR analysis

Total RNA was isolated from hypoxia-treated breast cancer cells by direct addition of TRIZoL reagent to the culture dishes. Total RNA (1ug) was then reverse transcribed using an oligo (dT) primer and reverse transcriptase using the advantage PCR kit according to manufacturer's instructions (BD Clontech, Palo Alto, CA). PCR amplification was performed as previously described[27] with gene specific primers HIF-1 α, (forward: 5′-CCAGATTCAGGATCAGACACCTAGTCCT-3′: reverse 5-′GCTCCATTCCATTCTGTTCACTAGATTTG-3′) uPA, (forward 5′-TGTGGCCAAAAGACTCTGAGGC-3′ reverse 5′-CTTGGTGTGACTGCGGATCCA-3′) uPAR (forward 5′-GAAGAACAGTGCCTGGATGTGGTGA-3′, reverse 5′-AGGTTTAGGTCCAGAGGAGAGTGCCTC-3′). 18S rRNA primers (forward: 5′-TTGGAGGGCAAGTCTGGTGCCAGCAGC-3′ reverse:5′ TCTGTCAATCCTGTCCGTGTCGGGCC-3′) were used as controls for PCR amplification under hypoxic conditions.

Preparation of cytosolic fractions

To determine whether hypoxia affects sub-cellular distribution of uPA/uPAR, cells which were incubated for (0–24 hrs) under hypoxia were harvested and used for cytosolic fractionation as described previously.[28] The protein content of cytosolic fractions were determined using Protein Assay Reagent Kit (Pierce Corp., Rockford, IL).

Western blot analysis

Equal amounts of cytoplasmic protein from various regiments were subjected to 10% SDS-PAGE and then transblotted onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membranes were blocked in TBS-TB buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl supplemented with 0.05% Tween-20; 0.05% BSA) containing 5% non-fat dry milk, and incubated with monoclonal antibodies to uPAR 3937 2 μg/ml, uPA, 3689 2 μg/ml, (American Diagnostica Inc, Greenwich, CT) and HIF-1α, 610958, 1:250 dilution, (BD Transduction Laboratories, Lexington, KY) followed by incubation with 1:5000 dilution of HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories. Inc, West Grove, PA). The reaction products were visualized using the ECL chemiluminescence detection kit (ECL; Perkin Elmer, Life Sciences Inc, Boston MA). To test for equal loading, the blots were stripped and reprobed with a monoclonal antibody to actin (1:5000 dilution, MAB1501, Chemicon, Temecula, CA).

In vitro invasion assay

To examine the effect of maspin on hypoxia-induced in vitro invasive potential of breast cancer cells invasion assay was performed using the membrane invasion culture system (MICS), as described previously.[29] Briefly, cells were treated under hypoxic and normoxic conditions in the absence or presence of 20 μg/ml neutralizing anti-uPAR antibody (MAB807, R&D systems, Minneapolis, MN), for up to 16 hrs. Subsequently, 5×10^4 cells/wells were seeded into the upper wells of the MICS chamber containing RPMI 1640 Mito+ serum-free media. After 24hrs, the cells that had invaded through the coated matrix into the lower wells were harvested. The percentage of invasion was calculated as the total number of invading cells/total number of cells seeded X 100.

Zymographic analysis of plasminogen activators

The effect of maspin on the enzymatic activity of cell surface associated and secreted uPA in breast cancer cells MDA-MB-231, MDA-MB-231-GFP-maspin and normal mammary epithelial cells1436N1 was examined by gelatin zymography. Cells were cultured in serum-free media in 35 mm tissue culture dishes and incubated under hypoxia for (0–24 hrs). Conditioned media (CM) was then collected and either stored at −80°C or used immediately. Ten microliters of CM and 8 μg of cytosolic proteins were mixed with sample buffer without β-mercaptoethanol and subjected to plasminogen-dependent gelatinolytic zymography as described previously.[30]

RESULTS

Maspin inhibits hypoxia-induced regulation of uPA/uPAR in invasive/metastatic breast cancer cells

Based on the reported studies, the induced level of transcription factor HIF-1α is a hallmark of hypoxic conditions.[9] We first attempted to examine the changes in HIF-1α expression both at the mRNA and protein level in MDA-MB-231 and MDA-MB-231-GFP-maspin cells following exposure to hypoxic conditions, and then tested the consequences on uPA/uPAR expression by RT-PCR and Western blot analysis.

We observed an induction of HIF-1α and uPAR at the mRNA level after 1 hr of exposure to hypoxia, which remained elevated for up to 24 hrs, whereas, no detectable changes in mRNA were observed for uPA (Fig. 1A and C). Interestingly, in the MDA-MB-231-GFP-maspin cells there were no detectable changes under hypoxic conditions in the mRNA levels of either HIF-1α or uPAR and uPA (Fig. 1B and D).

To examine whether mRNA changes were translated at the protein level, Western blot analysis was performed. This analysis indicated that hypoxia induced the protein expression of HIF-1 α and uPAR in the MDA-MB-231 cells reaching its maximum level by 8hrs (Fig. 2A and C). Quantification of uPAR protein bands by densitometry showed a 1–2 fold decrease in uPAR protein levels in cells transfected with maspin (Fig. 2B and C). With respect to uPA we observed that in MDA-MB-231 cells hypoxia induced expression of uPA by 1hr and reached its maximum level by 24 hr (Fig. 2D and F) with no detectable changes in the protein bands from maspin transfected cells (2E). Densitometric analysis showed that maspin was able to inhibit the uPA protein level by 2-fold (Fig. 2F) in MDA-MB-231-GFP-maspin cells under hypoxic conditions. However, the normal mammary epithelial cells 1436N1 revealed no significant increase in uPAR protein expression in hypoxic fractions as compared to controls, and we were unable to detect any expression of uPA protein under hypoxic and normoxic conditions, respectively (Fig. 2G).

Maspin affects hypoxia induced tumor cell invasion in vitro

Previous studies have shown that hypoxia increases the invasiveness of MDA-MB-231 cells, which can be abrogated by a function-blocking anti-uPAR antibody.[15,31] In the current study, we observed that maspin transfection of MDA-MB-231 significantly reduced the invasive potential of these cells, under both normoxic and hypoxic conditions (Fig. 3).

Maspin inhibits secreted uPA and cell surface associated uPA-uPAR complexes

UPA is an endogenous activator of the thrombolytic mediator plasminogen, however, in a tumor microenvironment its proteolytic activity could be directed towards the ECM, thus contributing to the degradation of ECM, dissemination of tumor cells, and subsequent tumor progression.[13] To assess the effect of maspin on the enhanced proteolytic activity of uPA under hypoxic conditions, we performed zymography in the presence of plasminogen, a specific substrate of uPA.

The zymographic data revealed activity bands at approximately 52–55 kDa in the cytosolic extract (Fig. 4A), representing cell associated uPA/uPAR complexes and in the serum-free conditioned media (Fig. 4C), representing secreted uPA. Our studies indicated that maspin transfection of MB-231 cells resulted in the reduction of both cell associated and secreated uPA activity under normoxic and hypoxic conditions. Interestingly, the results also indicate that the uPA activity in conditioned media under normoxia is about 2-fold higher as compared to cells exposed to hypoxic environment both in wild type MDA-MB-231 and maspin transfected MDA-MB-231-GFP-maspin cells.

By comparison analysis of the uPA activity in the cytosolic extract and serum free conditioned media from normal mammary epithelial cells 1436N1 showed extremely low levels of cell associated uPA activity and no detectable levels of secreted uPA (Fig. 4A and B). These data suggest that maspin in normal mammary epithelial cells as compared to metastatic cancer cells could downregulate uPA protein as well as activity levels.

DISCUSSION

Our studies indicate, for the first time, the ability of maspin (a serine proteinase inhibitor) to mitigate the hypoxia-induced uPA/uPAR expression in highly invasive breast cancer cells. Specifically, we have shown that inhibition of uPA complex by the tumor suppressor gene *maspin* reduced the effect of uPAR dependent activation of uPA and inhibited in vitro invasion of ECM. These observations further extend the reported studies that exposure of trophoblasts, human umbilical vein endothelial (HUVECs), colon and breast carcinoma cells to hypoxic conditions results in the upregulation of uPAR expression and increased in vitro

invasive activity[15,31,32] In addition, it further complements the reported tumor suppressive effects of maspin on breast cancer cell invasion by identifying its ability to target the uPA system in a hypoxic environment.

The production of pro-uPA and its subsequent activation to uPA by its interaction with uPAR is an important step in cancer cell invasion, as it is required for efficient activation of plasminogen to plasmin and ECM degradation.[32,33] Downregulation of both uPA and uPAR would be an efficient way to circumvent the possibility of uPAR-dependent, uPA mediated plasminogen activation.[16,35,36] In this regard, various approaches have been utilized to interfere with the expression and/or activity of uPA in tumor cells, including, the use of active site inhibitors, antibodies to uPA, oligonucleotide or RNA directed against uPA.[37–38] Other attempts to abrogate the uPA/uPAR interaction include the use of synthetic uPA-derived peptides encompassing the binding region of uPA to uPAR,[39] or recombinant souble uPA as scavenger for uPAR.[40] The catalytic activity of the uPA system can also be inhibited by its several natural inhibitors, including maspin, PAI-1 and PAI-2.[13,19] These inhibitors play distinct roles in tumor progression and PAI-1 has been shown to be specifically involved in the progression of cancer.[41] In contrast, maspin has been shown to reduce cell migration in vitro[23–24] and angiogenesiss in various cell types.[42] Furthermore, studies have shown that recombinant maspin has the ability to inhibit invasion and motility of mammary carcinoma cells in culture.[24–25] Additional studies from our laboratory have indicated that maspin suppresses breast cancer cell invasiveness by modulating integrin expression and altering Rac level and activity.[26]

Our data further substantiate the tumor suppressive role of maspin, by demonstrating that reexpression of maspin in highly invasive and metastatic MDA-MB-231 cells decreased the hypoxia-induced expression of both uPA/uPAR resulting in reduced in vitro invasive ability, thus confirming the significance of the uPA system underlying the invasive behavior of breast cancer cells under hypoxic conditions. We also observed that addition of a neutralizing uPAR antibody to MDA-MB-231 and MDA-MB-231-GFP-maspin cells inhibited the stimulatory effect of hypoxia on cellular invasion, indicating a functional relationship between maspin and the uPA system under hypoxic environment. Analysis of normal mammary epithelial cells 1436N1 (that express high levels of endogenous maspin) revealed little to no amount of uPA protein expression and enzymatic activity, suggesting the possibility that maspin in normal mammary epithelial cells could downregulate uPA protein levels.

Interestingly, the zymography data showed a dramatic decrease in the activity of secreated uPA after hypoxic treatment. Low levels of uPA activity have been reported under hypoxia in CM of MDA-MB-231 breast cancer cells and human microvascular endothelial cells (HMVECs).[15,43] Although the precise mechanism for this observation is unknown, it is likely that increased uPAR expression under hypoxia resulted in increased cell-surface bound uPA leading to its rapid internalization and depletion of uPA in the CM. This possibility is further supported by a previous study indicating that cell surface bound uPA induces plasminogen activation several fold when compared with that of unbound uPA.[35]

Although the tumor suppressive properties of maspin are consistent with those of protease inhibitors, the exact biological mechanism by which maspin exerts its tumor suppressive action is unclear. However, we speculate that maspin regulates the uPA system by inactivating uPA and enhancing the rate of uPA internalization and degradation. It is also likely that maspin inhibits the uPA system by affecting cell adhesion, either directly or indirectly based on maspin's ability to alter the expression profile of integrins, in particular $\alpha_5\beta_1$, in breast cancer cells.[45] Since uPAR is known to interact with $\alpha_5\beta_1$, this interaction

may lead to a reduction in uPA binding and a concomitant reduction in cell surface plasminogen activation, eventually inhibiting invasion and metastasis.[45]

In summary, we have demonstrated that a tumor suppressor gene *maspin* has the ability to inhibit the hypoxia-induced cell surface plasminogen activation affecting in vitro invasive activity in MDA-MB-231 breast cancer cell (Fig. 5). Since localized plasmin inhibition seems to be an essential route to inhibiting invasion; therefore, maspin is an interesting candidate for therapeutic development in the management of both tumor invasion and metastasis under hypoxic conditions.

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ABBREVIATIONS

uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
HIF-1α	hypoxia-inducible factor-1α
PAI-1 and 2	Plasminogen activator inhibitor 1 and 2
ECM	extracellular matrix
CM	conditioned media
ATF	amino-terminal fragment

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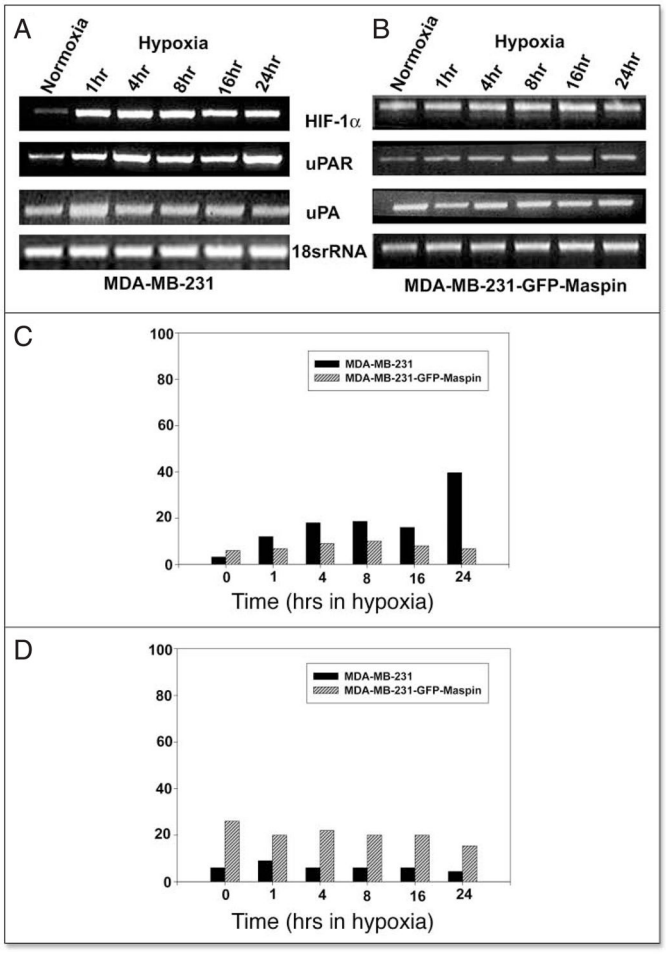

Figure 1.

Effect of maspin on uPA and uPAR expression under hypoxia. MDA-MB-231 (A) and MDA-MB-231-GFP-Maspin (B) were cultured under hypoxia for (0–24 hrs). Total RNA was then isolated and analyzed by RT-PCR using uPAR, uPA and HIF-1α specific primers. 18s rRNA primers were used as control for equal loading. The signals for uPA and uPAR mRNA were quantified by densitometric analysis and represented as the ratio of uPA: 18srRNA and uPAR:18s rRNA (C and D). Results shown in the lower panels are representatives of three independent experiments and were performed using Scion image analysis and Sigma plot.

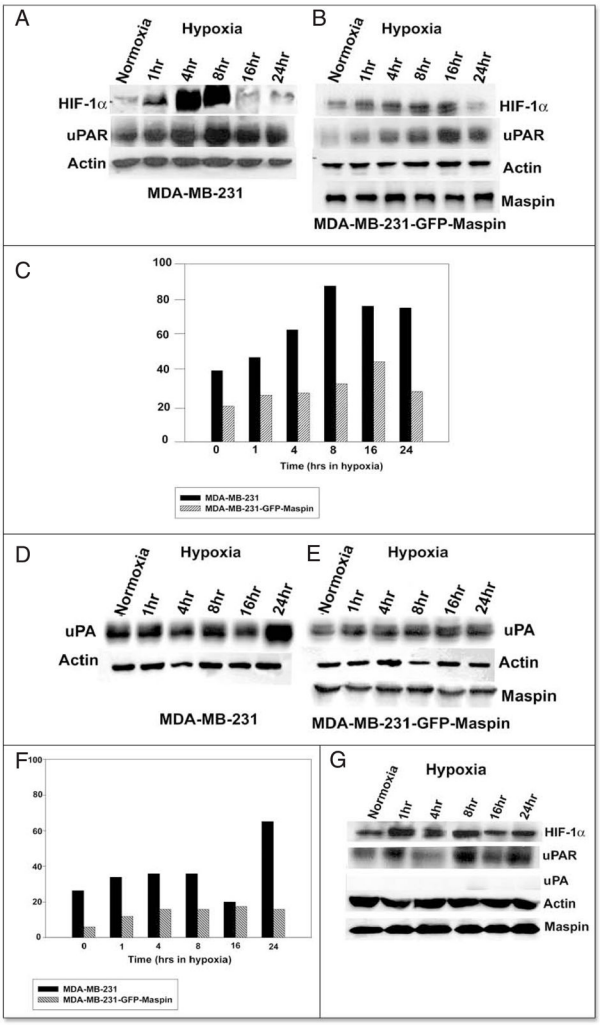

Figure 2.

Maspin inhibits the uPAR and uPA expression in MDA-MB- 231-GFP-Maspin cells. Western blot analysis of uPAR and uPA protein in MDA-MB-231 (A) MDA-MB-231-GFP-maspin (B) and normal mammary epithelial cells 1436N1 (C) cultured for (0–24 hrs) under hypoxia. Equal amounts (25 μg) of cytosolic protein was resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel, transblotted onto nitorcellulose membrane, and probed with anti-HIF-1α, uPAR and uPA monoclonal antibodies. Monoclonal antibody to actin was used as a control for equal loading. The data represents the densitometric analysis of results represented as the ratio of uPA: actin and uPAR:actin (C and F). Results shown in the lower panels were performed using Scion image and Sigma plot are representatives of three to five independent experiments.

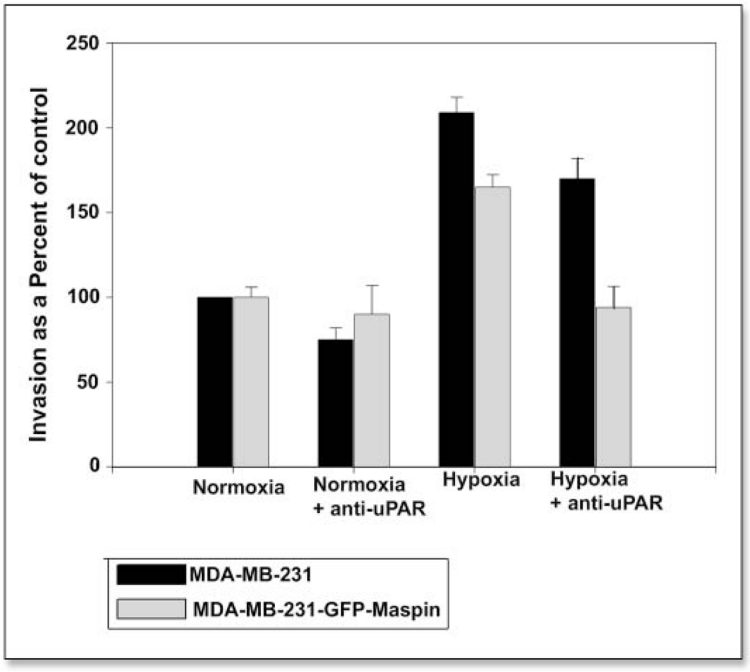

Figure 3.

Maspin inhibits hypoxia-induced in vitro invasion in human breast cancer cells MDA-MB-231. Equal amounts (50,000) cells/wells were seeded into the upper wells of the MICS chamber containing Mito+ serum-free media, following a 24 hr incubation under either hypoxia or normoxia in the absence or presence of 20 μg/ml anti-uPAR neutralizing antibody. After 24 hrs the cells in the bottom chamber were harvested and counted. The percentage of invasion was determined by counting the number of cells that migrated through a collagen 1V/laminin/gelatin-coated polycarbonate fiter in 24 hr/total number of cells seeded X 100). The Data are expressed as a percentage of normoxic cells and are representatives of 3–4 independent experiments.

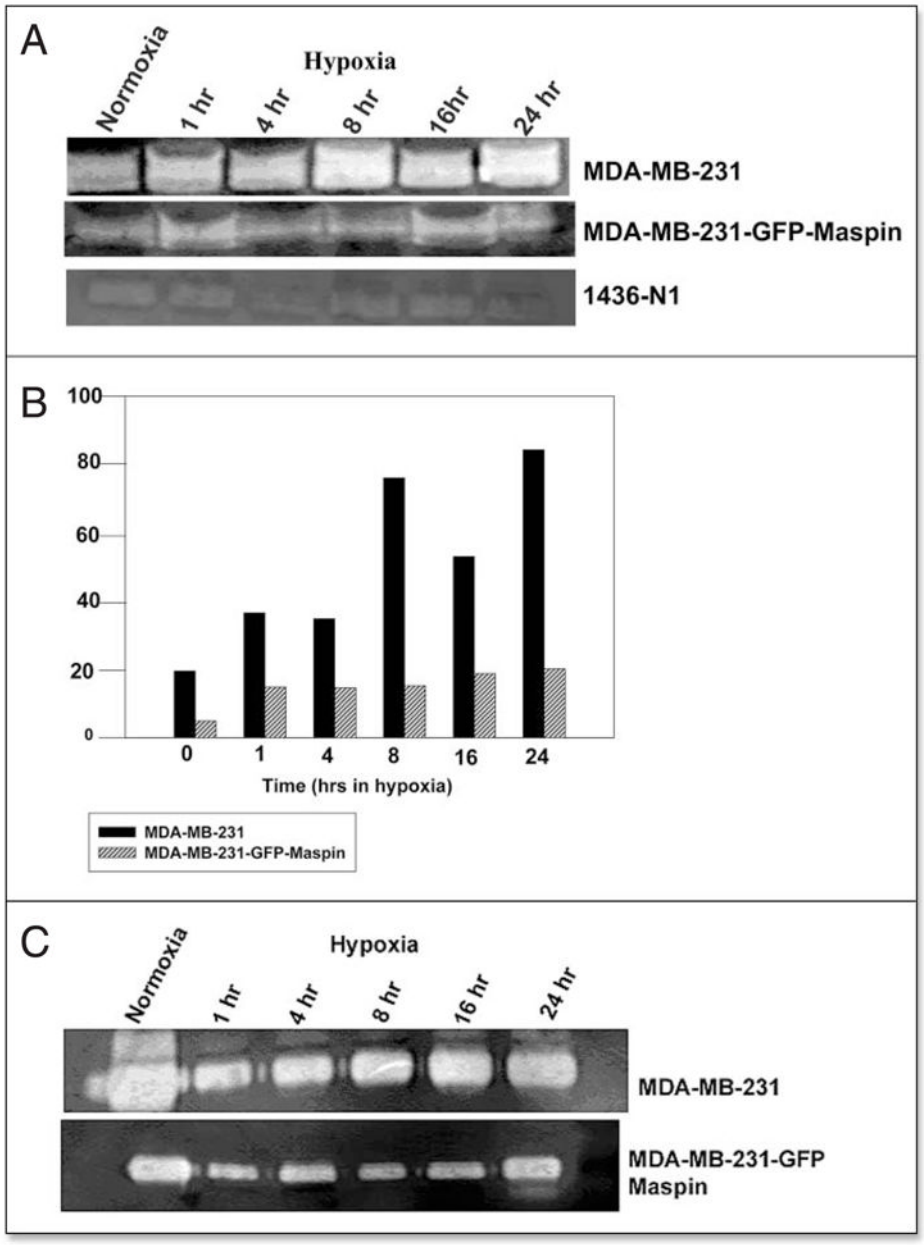

Figure 4.

Maspin inhibits the activity of cell associated and secreted uPA in MDA-MB-231 and MDA-MB-231-GFP-Maspin cells. UPA activity of (A) cell-associated and secreted (C) in breast cancer cells MDA-MB-231, MDA-MB-231 GFP-maspin and normal mammary epithelial cells 1436N1 under hypoxia at indicated times as determined by zymography. Clear bands at 50–55 kDa represent enzymatic activity of uPA due to the activation of plasminogen. Levels of uPA activity were quantified by densitometry employing scion image analysis and sigma plot (B and D). Results shown in the lower panels are representatives of three independent experiments.

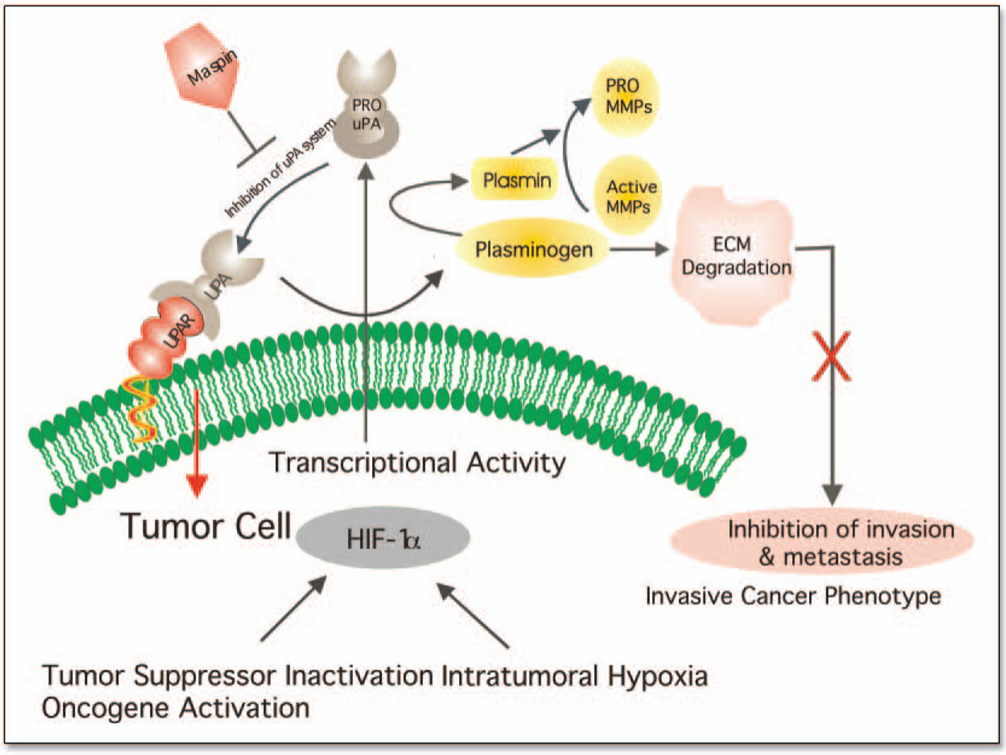

Figure 5.

Hypothetical model for the regulation of uPA system by maspin under hypoxia in breast cancer cells. Exposure of cancer cells to hypoxic environment leads to increased secreation of prouPA which then binds to its cellular receptor uPAR. This interaction between uPA-uPAR provides inducible, transient and localized cell surface proteolytic activity, required for tissue invasion, by enhancing the conversion of plasminogen to plasmin. Plasmin can either directly degrade basement ECM or activate other zymogen proteases such as procollagenase, resulting in increase invasive potential of tumor cells. In this model maspin regulates the uPA system by blocking the interaction of uPA with uPAR by binding to uPA and inactivating it. The maspin-uPA complex together with uPAR gets internalized and degraded. Thus, inhibiting migration, invasion and ultimately metastasis by the highly invasive and metastatic breast cancer cells MDA-MB-231.