Tyrosine phosphorylation plays a role in increasing maspin protein levels and its cytoplasmic accumulation

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ABSTRACT

Maspin is a tumor suppressor with many biological activities, multiple ligands and different subcellular localizations. Its underlying molecular mechanism remains elusive. We hypothesized that phosphorylation might regulate maspin localization and function. Using two-dimensional gel electrophoresis with different focusing power followed by Western blot we identified four different maspin forms with the same molecular weight (42 kDa), but different isoelectric points. Three of these forms were sensitive to acidic phosphatase treatment, suggesting that they are phosphorylated. Sodium peroxidovanadate treatment, a protein-tyrosine phosphatase inhibitor, resulted in a rapid increase in maspin protein levels and cytoplasmic accumulation. These data show that there are three different maspin tyrosine phosphoforms. Inhibition of tyrosine phosphatases increased maspin protein levels and leads to its cytoplasmic accumulation.
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1. Introduction

Maspin (SERPINB5) is a 42 kDa non-inhibitory serpin with tumor suppressor activity. Maspin is expressed in most epithelial tissues and presents multiple biological activities including inhibition of tumor growth and metastasis in vivo, modulation of cell adhesion in vitro, inhibition of cell migration and angiogenesis, regulation of apoptosis and oxidative stress response [1]. Maspin pleiotropic activities reflect its diversity of ligands and subcellular localization [1–7]. Different maspin PTMs have been reported, including phosphorylation [8,9], nitrosylation and intramolecular disulfite bonds [10], suggesting that PTMs regulate maspin function. Initial studies reported a strong maspin tumor suppressor activity [11], in agreement with decreased maspin expression in tumor progression of different tumor types [11,12]. Conversely, others have shown a poor prognosis in maspin-expressing tumors [13–15]. Recent reports found that cytoplasmic maspin was associated with poor prognosis, whereas nuclear maspin localization was indicative of a less aggressive lesion [16], indicating that maspin

subcellular localization, rather than expression levels, correlates with tumor progression. Using the MFC-10A model, a non-transformed human mammary epithelial cell line which endogenously expresses maspin, we identified four maspin forms, three of them were found phosphorylated. Cell treatment with sodium peroxidovanadate, a tyrosine phosphatase inhibitor, increased maspin protein levels, which accumulated predominantly in the cytoplasm. Interestingly, high levels of acidic maspin were detected in the AsPC-1 pancreatic tumor cell line, which expresses maspin predominantly in the cytoplasm [15]. These results suggest that tyrosine phosphorylation may play a role in maspin protein levels and correlates with cytoplasmic accumulation.

2. Materials and methods

2.1. Cell culture

AsPC-1 and MCF-10A cells were cultivated as described [6,15]. For sodium peroxidovanadate treatment, equal volumes of 20 mM sodium orthovanadate and 20 mM hydrogen peroxide were freshly mixed and incubated for 30 min at room temperature. Two hundred micrograms per milliliter of catalase was added to decompose unreacted hydrogen peroxide. Cell medium was changed 2 h before peroxidovanadate treatment (25 μM) for indicated periods of time. Control cells were incubated with the same solution without peroxidovanadate [17].

2.2. Cell lysates

For IEF, 90–100% confluent MCF-10A or AsPC-1 cultures were extracted in 8 M urea, 4% CHAPS, 40 mM DTT, 1X protease inhibitor cocktail (Sigma # P2850), 1X phosphatase inhibitor cocktail (Sigma # P2714) and cleared by centrifugation (whole cell extract). In order to prepare an extract which is compatible with acidic phosphatase treatment and IEF, we used the 2D Sample Prep Kit for Nuclear Proteins (Pierce # 89863) according to manufacturer's instructions. Acidic phosphatase solution (Sigma) was freshly prepared in 40 mM PIPES pH 6.0, 1 mM DTT, 20 μg/ml aprotinin and 10 mM PMSF. Protein extracts were incubated with 100 μg/ml of acidic phosphatase (Sigma) for 30 min at 37 °C. For immunoprecipitation, whole cell extract were prepared in modified RIPA buffer [6] and cleared by centrifugation. Protein concentration was determined by the Bradford assay (Bio-Rad).

2.3. 2D-SDS-PAGE

90–100% confluent MCF-10A cultures grown in complete medium were used in all the experiments. Protein samples (100–2000 μg) were applied onto 3–10 (11 cm), 4–7 (13 cm) (GE Healthcare) or 4.7–5.9 (17 cm) (Bio-Rad) linear immobilized pH gradient strips. Strips were rehydrated for 16 h at room temperature. IEF was performed on an IPGphor III apparatus (GE Healthcare) at 17 kV h. For the second dimension, strips were incubated at room temperature for 20 min in equilibration buffer (6 M urea, 2% SDS, 50 mM Tris–HCl, pH 6.8, 30% glycerol and 0.001% bromophenol blue with 2% DTT), followed by incubation with 4% iodoacetamide in equilibrium buffer for 20 min. The second dimension was performed in vertical 12% SDS-PAGE (Ettan DALT large vertical electrophoresis, GE Healthcare). Proteins were transferred to PVDF membrane and analyzed by Western blot with monoclonal anti-maspin (Millipore MAB 4035).

2.4. Immunoprecipitation and Western blot

Two milligrams of MCF-10A RIPA buffer lysates were incubated with rabbit anti-maspin (BD Pharmingen) or anti-phosphotyrosine (Millipore, clone PY20) overnight at 4 °C. Protein G-coupled beads were added for 1 h at 4 °C under agitation; beads were washed with ice-cold RIPA buffer and boiled for 5 min in Laemmli buffer under reducing conditions. Immunoprecipitated material was separated in 12% SDS-PAGE gels, transferred to PVDF membrane and probed with monoclonal anti-maspin and anti-phosphotyrosine. Other antibodies used in this study were anti-GAPDH (Advanced Immuno Chemical, Inc.) and anti-α-tubulin (Sigma). Appropriate secondary antibodies were added and immunodetection was performed by chemiluminescence and exposure of membranes to X-ray films.

2.5. Immunofluorescence

Sodium peroxidovanadate treatment was performed as described in the previous section. After treatment cells were fixed with 2% paraformaldehyde in PBS for 20 min at room temperature. The fixed cells were permeabilized with 0.05% Triton X-100 in blocking buffer (5% goat serum in PBS) for 1 h at room temperature. Cells were then incubated in 1:100 diluted monoclonal anti-maspin (BD Pharmingen) in blocking buffer and Alexa-Fluor 568 anti-mouse secondary antibody. Cells were counterstained with 0.1 mg/ml Hoechst dye to visualize the nuclei. Images were obtained with an Axiophot widefield fluorescence microscope using a 63× PlanApo 1.4 NA objective (Carl Zeiss). Images were acquired using a digital CCD monochromatic camera (CoolSnap HQ2, Photometrics Inc., Tucson, AZ, USA). The microscope and all devices

were controlled by Metamorph Premier 7.6 software (Molecular Devices, Sunnyvale, CA, USA). Fluorescence intensity and localization were measured using ImageJ public domain software.

3. Results and discussion

Maspin phosphorylation has been previously described in mammary and corneal epithelial cells [8,9]. Whereas multiple serine sites have been identified, maspin tyrosine phosphorylation and function has not been explored yet. To initially verify if maspin is phosphorylated on tyrosine residues in MCF-10A cells, whole cell lysates were immunoprecipitated with anti-maspin and anti-phosphotyrosine and analyzed by immunoblot with anti-maspin and anti-phosphotyrosine (Fig. 1). A strong maspin band was detected in the material immunoprecipitated by anti-phosphotyrosine, as well as a weaker band (Fig. 1, lane 3, arrow and arrowhead, respectively). The high molecular weight form was not pursued in this study. We were unable to detect phosphotyrosines in the material immunoprecipitated by different monoclonal and polyclonal maspin antibodies (data not shown), even though maspin could be efficiently immunoprecipitated (Fig. 1, lane 2). This result suggests that a small fraction of the maspin intracellular pool is phosphorylated on tyrosine residues under our experimental condition. Since addition of a phosphate group adds a negative charge to the protein, we reasoned we would be able to detect different maspin forms by IEF followed by Western blot. For this purpose 100 μg of whole cell extract were analyzed on an 11 cm pH 3–10 linear gradient IPG strip. A predominant spot (Fig. 2A, arrow) next to a possible precursor of an independent spot (Fig. 2A, arrowhead) was detected with anti-maspin. By this first analysis maspin pI was estimated to be around 5.65, which approaches a previous prediction [8]. In a second analysis 700 μg was applied on a 13 cm IPG strip with a narrower pH gradient (pH 4–7). Two distinct spots could be detected, a strong one and a more acidic less abundant spot (Fig. 2B, arrow and arrowhead, respectively). In order to increase the relative focusing power and reach the finest possible resolution, 2 mg of whole cell extract were applied to a 17 cm strip with a micro-range gradient (pH 4.7–5.9). Four different spots were detected (Fig. 2C, arrows). In order to confirm these spots were due to phosphorylation, maspin was immunoprecipitated from whole cell extract and immunoprecipitated material was analyzed by 2D-SDS-PAGE followed by Western blot with anti-phosphotyrosine. In at least four different attempts using different
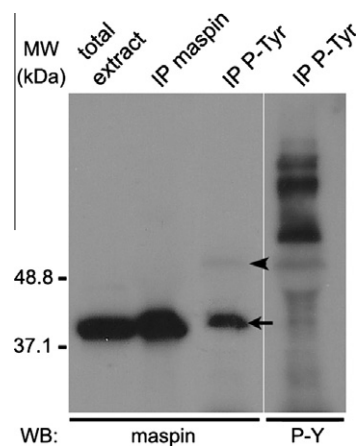

Fig. 1. Maspin is phosphorylated on tyrosine residues in MCF-10A cells. Five hundred micrograms of whole cell extract was analyzed by immunoprecipitation followed by Western blot with the indicated antibodies. Fifty micrograms of whole cell extract was loaded in the first lane of the gel. Arrow and arrowhead indicate maspin band and an unidentified molecule, respectively.
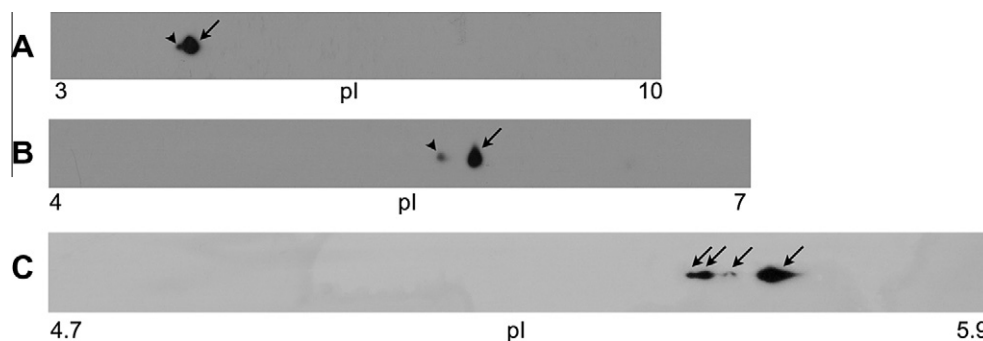
Fig. 2. Identification of different maspin forms in MCF-10A cells. Whole cell extracts were analyzed by 2D-SDS-PAGE followed by Western blot with anti-maspin. Isolectric focalizations were carried out in three different IPG strips: (A) 100 µg protein/11 cm pH 3–10; (B) 700 µg/13 cm pH 4–7 and (C) 2000 µg/17 cm pH 4.7–5.9. Arrows and arrowhead indicate different maspin forms.
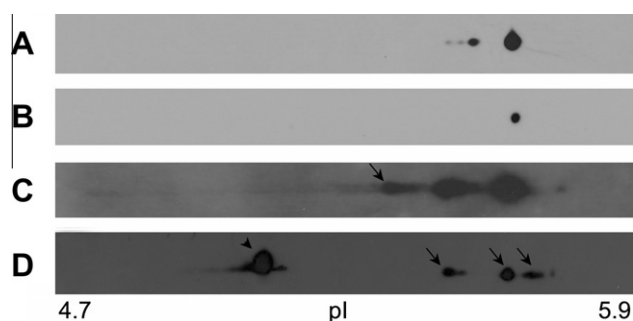

Fig. 3. Maspin phosphorylated forms. Two milligrams of protein extracts were analyzed by 2D-SDS-PAGE using 17 cm pH 4.7–5.9 IPG strips followed by Western blot with anti-maspin. (A) MCF-10A nuclear extracts; (B) MCF-10A nuclear extracts treated for 30 min with acidic phosphatase; (C) MCF-10A cells were treated with 25 µM sodium peroxidovanadate for 90 min before whole cell extraction. Arrow indicates maspin phosphoforms not observed in previous analyses; (D) AsPC-1 total cell extracts. Arrows indicate maspin spots which resemble spots detected in 3C. Arrowhead indicates a strong acidic maspin form.

Fig. 4. Peroxidovanadate leads to rapid increase in maspin levels in MCF-10A cells. (A) Cells were treated with 25 µM sodium peroxidovanadate for 15, 45 and 90 minutes and 50 µg of whole cell extract was analyzed by Western blot (upper panel). The membrane was reprobed with anti-α-tubulin for a loading control (lower panel). (B) Maspin signal was quantified and normalized to the level of α-tubulin.

antibodies, we were never able to detect maspin spots with anti-phosphotyrosine (data not shown), in accordance with what we observed in experiment shown in Fig. 1. A previous study could not detect phosphotyrosine in corneal epithelial cell lysates immunoprecipitated with anti-maspin either [8]. Therefore, we suspect maspin tyrosine phosphoforms are not efficiently immunoprecipitated by the commercially available maspin antibodies. Since maspin is present in multiple cellular compartments and it is also detected in the Triton X-100 insoluble cytoskeleton fraction [6], cells lysates were prepared in a strongly denaturing and chaotropic solution containing 8 M urea (see Section 2), to reassure that maspin associated with the different cellular components would be extracted. However, this extraction solution precludes the phosphatase treatment of the extract. To circumvent this experimental limitation, we acquired a kit for nuclear extraction which is compatible with both IEF and acidic phosphatase treatment (described in Section 2). Two milligrams of nuclear extract was analyzed by 2D-SDS-PAGE followed by Western blot with anti-maspin. Four maspin spots were detected in this fraction (Fig. 3A), which present a similar distribution pattern observed in whole cell extract (Fig. 2C). Acidic phosphatase treatment of the extract followed by similar analysis completely abolished three of the four spots initially detected (Fig. 3B), indicating that these three spots are phosphorylated maspin. To further support the phosphorylation of maspin forms and confirm that it occurs on tyrosine residues, MCF-10A cells were treated with sodium peroxidovanadate for 90 min. Whole cell extracts were prepared and analyzed by 2D-S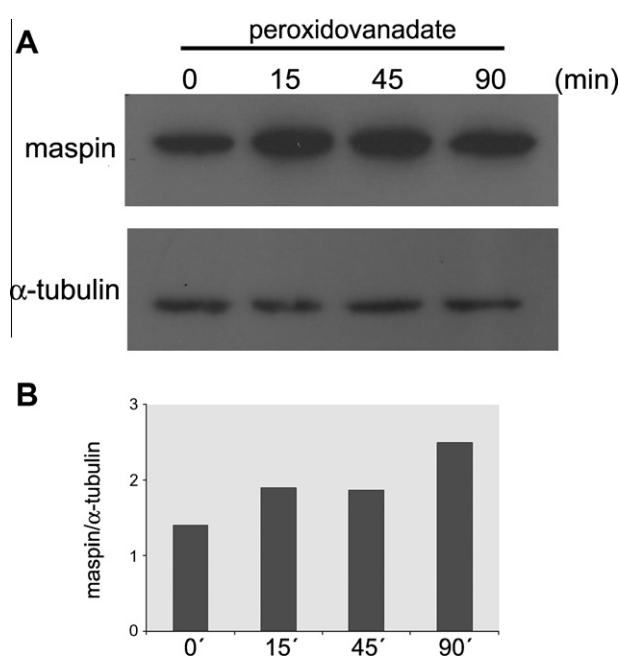DS-PAGE followed by Western blot with anti-maspin. We observed a strong increase in maspin spots (Fig. 3C), indicating that maspin is phosphorylated on tyrosine, and an increase on overall maspin levels as well. Interestingly, we observed a smeared spot which was not apparent in the untreated extracts (Fig. 3C, arrow), indicating that there might be more maspin tyrosine phosphoforms which are present in very low levels in the cells. In addition, this result suggests that maspin tyrosine phosphorylation might be hold in check by tyrosine phosphatases.

To gain some insight into the biological role of maspin tyrosine phosphorylation, maspin protein levels and subcellular localization were investigated. MCF-10A cells were treated with peroxidovanadate for 15, 45 and 90 min and whole cell extract was analyzed by Western blot. A 35% increase in maspin protein levels was detected after 15 min of peroxidovanadate treatment (Fig. 4A). This level did not change after 45 min and further increased 77.7% in 90 min (Fig. 4B). The rapid increase in maspin

levels upon peroxidovanadate treatment is in agreement with the increase in maspin spots (Fig. 3C). In order to visualize maspin increment, MCF-10A cells were treated with peroxidovanadate for 90 min, fixed and processed for immunofluorescence with anti-maspin monoclonal antibody. Maspin total levels significantly increased upon peroxidovanadate treatment (Fig. 5A and B), in good agreement with the Western blot and the IEF (Figs. 4A and 3C, respectively). Interestingly, quantification of the cytoplasmic and nuclear maspin revealed that there is 1.8 times more maspin in the cytoplasm than in the nucleus of peroxidovanadate-treated cells, whereas maspin appears equally distributed between these two compartments in control cells (Fig. 5B). These results suggest that tyrosine phosphorylation plays a role in maspin protein levels and leads to cytoplasmic accumulation.

Because growing evidence indicates that in tumor cells, greater cytoplasmic than nuclear maspin correlates with tumor progression, we looked at maspin spots in the AsPC-1 pancreatic tumor cell line, which expresses maspin mostly in the cytoplasm [15]. AsPC-1 whole cell extracts were prepared and analyzed by 2D-SDS-PAGE followed by Western blot with anti-maspin. Three maspin spots were detected (Fig. 3D, arrows), which resembles those found in peroxidovanadate-treated MCF-10A cells (compare spots in 3C and 3D). Interestingly, a more acidic maspin form (estimated pI ≈ 5.2) was strongly expressed in AsPC-1 cells (Fig. 3D, arrowhead). The similarity between the spot pattern found in AsPC-1 and in peroxidovanadate-treated MCF-10A cells

suggests that maspin is phosphorylated on tyrosine in AsPC-1 cells as well. This observation further supports the correlation between maspin phosphorylation and cytoplasmic accumulation and indicates a possible link between maspin tyrosine phosphorylation and tumor progression. Phosphorylation of nuclear localization and nuclear export signals regulates nucleocytoplasmic trafficking, affecting gene transcription, cell cycle and proliferation [18]. Identification of the phosphorylated residues and site-directed mutagenesis will help to elucidate if maspin phosphorylation plays a causative role in maspin nuclear localization. The strong increase in maspin phosphoforms upon peroxidovanadate treatment suggests that intracellular maspin is predominantly phosphorylated on tyrosine residues (Fig. 3B). In agreement with our result, a recent study reported that maspin tyrosine phosphorylation was found in the intracellular compartment of corneal epithelial cells, but not in the secreted form [8]. These results may indicate that maspin phosphorylation on serine/threonine versus tyrosine could play a role in maspin secretion, as well as subcellular localization. Clinical studies suggest that maspin nuclear localization is indicative of better prognosis [19,20]. In agreement with these observations, exclusion of maspin from the nucleus abrogates its tumor suppressor activity [21]. This study is the first to address the biological role of maspin tyrosine phosphorylation. In addition, it indicates a possible mechanism for maspin subcellular localization, which can be a promising therapeutic target.
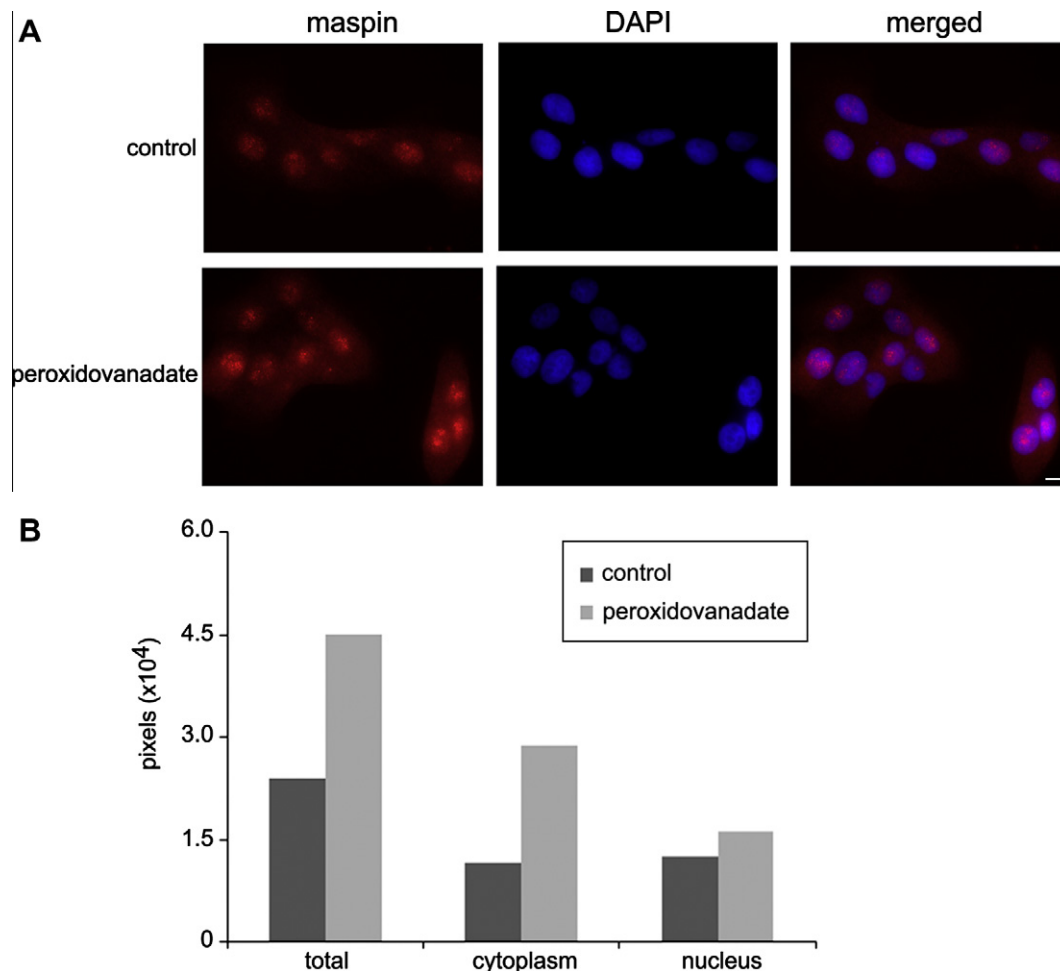

Fig. 5. Maspin cytoplasmic accumulation in peroxidovanadate-treated MCF-10A cells. (A) Cells were treated with 25 μM sodium peroxidovanadate for 90 min, fixed and processed for immunofluorescence with anti-maspin monoclonal antibody. Nuclei were counterstained with Hoechst dye. (B) Total maspin as well as cytoplasmic and nuclear maspin were quantified and expressed in arbitrary units. Scale bars – 10 μm.

Acknowledgements

References

[1] Bailey, C.M., Khalkhali-Ellis, Z., Seftor, E.A. and Hendrix, M.J. (2006) Biological functions of maspin. J. Cell. Physiol. 209, 617–624.

[2] Endsley, M.P., Hu, Y., Deng, Y., He, X., Warejcka, D.J., Twining, S.S., Gonias, S.L. and Zhang, M. (2011) Maspin, the molecular bridge between the plasminogen activator system and beta1 integrin that facilitates cell adhesion. J. Biol. Chem. 286, 24599–24607.

[3] Li, X., Yin, S., Meng, Y., Sakr, W. and Sheng, S. (2006) Endogenous inhibition of histone deacetylase 1 by tumor-suppressive maspin. Cancer Res. 66, 9323–9329.

[4] Blacque, O.E. and Worrall, D.M. (2002) Evidence for a direct interaction between the tumor suppressor serpin, maspin, and types I and III collagen. J. Biol. Chem. 277, 10783–10788.

[5] Bailey, C.M. et al. (2005) Mammary serine protease inhibitor (maspin) binds directly to interferon regulatory factor 6: identification of a novel serpin partnership. J. Biol. Chem. 280, 34210–34217.

[6] Cella, N., Contreras, A., Latha, K., Rosen, J.M. and Zhang, M. (2006) Maspin is physically associated with [beta]1 integrin regulating cell adhesion in mammary epithelial cells. FASEB J. 20, 1510–1512.

[7] Yin, S., Li, X., Meng, Y., Finley Jr., R.L., Sakr, W., Yang, H., Reddy, N. and Sheng, S. (2005) Tumor-suppressive maspin regulates cell response to oxidative stress by direct interaction with glutathione S-transferase. J. Biol. Chem. 280, 34985–34996.

[8] Narayan, M., Mirza, S.P. and Twining, S.S. (2011) Identification of phosphorylation sites on extracellular corneal epithelial cell maspin. Proteomics 11, 1382–1390.

[9] Odero-Marah, V.A., Khalkhali-Ellis, Z., Schneider, G.B., Seftor, E.A., Seftor, R.E., Koland, J.G. and Hendrix, M.J. (2002) Tyrosine phosphorylation of maspin in normal mammary epithelia and breast cancer cells. Biochem. Biophys. Res. Commun. 295, 800–805.

[10] Nawata, S., Shi, H.Y., Sugino, N. and Zhang, M. (2010) Evidence of post-translational modification of the tumor suppressor maspin under oxidative stress. Int. J. Mol. Med. 27, 249–254.

[11] Zou, Z. et al. (1994) Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science 263, 526–529.

[12] Heighway, J. et al. (2002) Expression profiling of primary non-small cell lung cancer for target identification. Oncogene 21, 7749–7763.

[13] Smith, S.L., Watson, S.G., Ratschiller, D., Gugger, M., Betticher, D.C. and Heighway, J. (2003) Maspin – the most commonly-expressed gene of the 18q21.3 serpin cluster in lung cancer – is strongly expressed in preneoplastic bronchial lesions. Oncogene 22, 8677–8687.

[14] Umekita, Y. and Yoshida, H. (2003) Expression of maspin is up-regulated during the progression of mammary ductal carcinoma. Histopathology 42, 541–545.

[15] Maass, N., Hojo, T., Ueding, M., Luttges, J., Kloppel, G., Jonat, W. and Nagasaki, K. (2001) Expression of the tumor suppressor gene maspin in human pancreatic cancers. Clin. Cancer Res. 7, 812–817.

[16] Goulet, B., Chan, G., Chambers, A.F. and Lewis, J.D. (2012) An emerging role for the nuclear localization of maspin in the suppression of tumor progression and metastasis. Biochem. Cell Biol. 90, 22–38.

[17] Posner, B.I. et al. (1994) Peroxovanadium compounds. A new class of potent phosphotyrosine phosphatase inhibitors which are insulin mimetics. J. Biol. Chem. 269, 4596–4604.

[18] Nardozzi, J.D., Lott, K. and Cingolani, G. (2010) Phosphorylation meets nuclear import: a review. Cell Commun. Signal 8, 32.

[19] Mohsin, S.K., Zhang, M., Clark, G.M. and Craig Allred, D. (2003) Maspin expression in invasive breast cancer: association with other prognostic factors. J. Pathol. 199, 432–435.

[20] Sood, A.K. et al. (2002) The paradoxical expression of maspin in ovarian carcinoma. Clin. Cancer Res. 8, 2924–2932.

[21] Goulet, B. et al. (2011) Nuclear localization of maspin is essential for its inhibition of tumor growth and metastasis. Lab. Invest. 91, 1181–1187.