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Tumor Suppressor Maspin as a Rheostat in HDAC Regulation to Achieve the Fine-Tuning of Epithelial Homeostasis

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Abstract

Maspin, a class II tumor suppressor, is often downregulated during tumor progression and its depletion from the nucleus is associated with poor prognosis. Recently, we reported that reintroduction of maspin is sufficient for redifferentiation of prostate cancer cells to epithelial phenotype, a reversal of epithelial-to-mesenchymal transition. We have linked this effect of maspin with its ability to directly inhibit HDAC1, thereby influencing the acetylation state of transcription factors and other proteins. Maspin overexpression leads to changes in the expression level of a large number of proteins and these changes are often microenvironment specific. In this review, we summarize the epigenetic effects of maspin and provide comprehensive bioinformatic analysis of microarray-derived gene expression changes caused by maspin in different microenvironments. The analysis was performed on multiple levels, including identification of statistically enriched gene ontology groups, detection of overrepresented transcription factors binding sites in promoters of differentially expressed genes, followed by searching for key nodes of regulatory networks controlling these transcription factors. The results are consistent with our hypothesis that maspin serves as an endogenous regulator of HDAC activity and suggest that the effect of maspin is primarily mediated by TGF β , β -catenin/E-cadherin pathways, and network key nodes such as Abl kinase, p62, IL1, and caspases 6 and 8.

Keywords

maspin; HDAC; tumor suppressor; re-differentiation; epigenetic regulation; E-cadherin; MEF2; Nanog; TGF β ; Abl

I. INTRODUCTION

Carcinogenesis and tumor progression are a continuum of genetic and epigenetic changes that drifting away from normal epithelial homeostasis. A summary of 852 studies on cancer genetics led to a surprising conclusion that among those driver genetic changes, there were more tumor suppressors than oncogenes.¹ In principle, a tumor suppressor contributes to tumorigenesis and tumor progression when it is lost. Practically, tumor suppressors can be divided into two classes. While class I tumor suppressors include deleted and mutated genes, class II tumor suppressors are those that are silenced by epigenetic or biochemical mechanisms. Maspin is an epithelial-specific class II tumor suppressive gene that is epigenetically downregulated in tumor progression.^{2,3} In experimental tumor models, maspin has been shown to inhibit tumor growth, restore epithelial differentiation, block invasion and metastasis *in vivo*, and sensitize tumor cells to drug-induced apoptosis.^{4–7}

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Interestingly, on apoptosis-inducing drug treatment, maspin can be upregulated in prostate epithelial cells.^{8,9}

Based on the general similarity of maspin sequence to serine protease inhibitors, and the evidence that the loss of maspin conferred a gain of function in tumor invasion and metastasis, we made a decade-long effort to investigate the possibility that maspin may inhibit a serine protease. Our investigation led to an unexpected conclusion: maspin acts as a serine protease inhibitor-*like* molecule to antagonize serine protease-*like* enzymes or zymogens.¹⁰ Furthermore, for reasons that are under investigation in our lab, maspin, a protein without specific address signals, can be partitioned to the nucleus, cytoplasm, cell membrane, and extracellular space.¹¹ The patterns of molecular interactions of maspin seem to vary in different subcellular compartments. We were the first to report that (i) secreted endogenous maspin binds and inhibits single-chain tissue type plasminogen activator (scfPA, a zymogen) that is bound to fibrin or fibrinogen,¹² (ii) extracellular maspin specifically binds and inhibits pro-urokinase type plasminogen activator (pro-uPA, a zymogen) that is associated with cell surface-anchored uPA receptor (uPAR),^{13,14} and (iii) intracellular maspin specifically interacts and inhibits histone deacetylase 1 (HDAC1).¹⁵ Although maspin in each subcellular compartment has a distinct role and biological consequences, the compartmentalized functions of maspin all seem to point to the direction of tumor suppression.

HDACs regulate gene expression and other cellular functions by deacetylating the lysine residues in the substrate proteins including histones, and are among the most promising targets for anticancer agent development. It is important to note that despite the clinical significance of HDAC inhibition and the advancement in the development of pharmacological HDAC inhibitors, how HDAC activity is endogenously regulated is largely unknown.

To our knowledge, maspin is the only endogenous polypeptide HDAC1 inhibitor identified thus far. HDAC1 is the most abundant class I HDAC in mammalian cells that is upregulated in many types of cancer including prostate cancer.¹⁶ On the basis of a homology model of HDAC1 structure, the catalytic domain of HDAC1 resembles that of serine proteases. Structural considerations of maspin, on the other hand, predict that maspin may prefer to bind a pseudoserine protease. While its reactive center loop can generally fit into a serine protease catalytic pocket, it cannot undergo proteolysis-induced conformational changes as a classical serine proteinase inhibitor.^{17,18} Data from maspin site-directed mutagenesis suggested that the Arg340 residue at the critical P₁P₁' peptide bond of maspin RCL may directly compete against the acetylated lysine in an HDAC substrate and bind to the catalytic site of HDAC.¹³ The inhibitory maspin/HDAC1 interaction may be the molecular basis for the tumor suppressive effects of maspin and the correlation between maspin, especially nuclear localization of maspin, and better prognosis observed in variety of cancers.^{19–21} It is tempting to postulate that the HDAC-mediated protein deacetylation could be controlled or fine-tuned by maspin. Thus, maspin may have a unique and central role in regulating the protein expression equilibrium in the cell. Consistent with this hypothesis, the overall maspin-induced gene expression profile supports the role of maspin as an endogenous HDAC1 inhibitor, and is further consistent with our earlier incidental findings that maspin expression increased the expression of p21 and cytokeratin 8 and 18, and decreased the expression of VEGF uPA and uPAR.^{13,20,–25} The inhibitory interaction between maspin and HDAC1 is also supported by cell biological and biochemical evidence.

Taking advantage of human prostate cancer cell line DU145 that does not express maspin, we examined the effect of maspin transfection on tumor progression and gene expression. Maspin transfected cells, versus the mock transfected control, displayed a progressively

more differentiated epithelial phenotype through monolayer culture on plastic dish (2D), 3D culture in collagen I matrix to xenograft tumors in the Scid-Hu model for human prostate cancer bone metastasis (*in vivo* bone tumor).²⁶ The maspin-induced tumor redifferentiation is progressively more extensive throughout the 2D→3D→*in vivo* bone tumor models, accompanied by increased expression and polarized subcellular localization of the epithelial differentiation marker E-cadherin.²⁶ These data support a role of maspin in controlling epithelial homeostasis, especially when tumor cells are grown in environments that closely resemble the *in vivo* microenvironment. The utilization of our established 2D, 3D, and *in vivo* bone tumor models that consistently support the biological function of maspin in reprogramming tumor cells for differentiation allows us to extrapolate the central pathways and functions that are regulated by maspin, as well as the maspin-dependent cellular response to changes of tumor microenvironments. Microarray profiles of gene expression were obtained to investigate these possibilities.²⁶ Briefly, genome-wide RNA microarray of prostate cancer cell line DU145 transfected with maspin (M7) or mock plasmid (Neo) identified 31 commonly upregulated genes and 29 commonly downregulated genes by maspin in all three experimental systems (2D, 3D, and *in vivo* enograft bone tumor). Additional maspin-induced changes in gene expression specifically associated with the experimental cellular/tumor microenvironments were also identified.²⁶

These profiles reveal the molecular events and context reprogrammed by maspin toward epithelial differentiation, but do not automatically specify the pathways that are controlled by maspin. In an effort to decipher the cellular regulatory circuitry regulated by maspin, we employed a comprehensive bioinformatic approach utilizing gene regulation analysis tools TRANSFAC (Ref. 27) and Ex-Plain (Ref. 28) developed by Biobase. Our analysis included ontology search, search for reported and putative transcription factor binding sites, and identification of key nodes and clusters in regulatory cascades conveying transcription regulation by maspin. In this review, we summarize our findings and introduce a new paradigm for the regulation of core cellular processes by maspin.

II. THE EFFECT OF MASPIN ON CELLULAR PROCESSES AND FUNCTIONS

To decipher collective biological functions associated with changes of gene expression profile, up- or downregulated genes obtained in microarray experiments are routinely tested for enrichment of gene ontology (GO) categories. For over a decade, GO (Ref. 29) provides conventional ontological classification of genes by biological process, molecular function, and cellular component. Recently, more parameters were added to include posttranslational modifications, protein domains, and involvement in diseases. In our study, to systematically assess major functional consequences of maspin-mediated gene expression, we used a one-tailed Fisher test³⁰ to evaluate statistical significance ($p = e^{-7}$) of the GO enrichment in model-specific +/- maspin (M7/Neo) data sets, and in the lists of commonly or uniquely up- or downregulated genes across different models (2D, 3D, and *in vivo*). In general, maspin expression induces specific and significant changes in protein expression patterns. Many of these changes are highly relevant for carcinogenesis. The most redundant and statistically significant GO categories are shown in Fig. 1.

Interestingly, more GO categories are downregulated than upregulated by maspin, and the statistical significance of the downregulated categories is, on average, higher. This finding was intriguing, given the fact that the numbers of up- and downregulated genes were different among our experimental models, yet similar within each model. It is, however, perfectly comprehensible if one assumes that the significant, or even major, influence of maspin on gene expression is by inhibiting HDAC1. As HDAC1 deacetylates histons (among many other targets), its inhibition must result in upregulation of a large number of functionally unrelated proteins. As the result of co-upregulation of unrelated proteins, less

GO categories are specifically influenced, as mentioned above. In addition, HDAC inhibition was reported to suppress DNMT1, leading to DNA demethylation and further upregulation of expression of many genes.³¹ Indeed, we have shown that the inhibition of HDAC1 by maspin led to demethylation of glutathione s-transferase pi, a gene commonly silenced in early progression of human prostate cancer.¹³

Functionally, the most affected by maspin is the expression of secreted proteins: extracellular region and extracellular matrix proteins are significantly enriched in both upregulated and downregulated gene sets among different model systems. The glycoproteins is the most significantly enriched category of upregulated genes. This finding is in line with our observation that maspin increased the deposition of extracellular matrix and downregulated expression of metalloproteinases.^{26,32} Interestingly, the protease category is significantly more enriched in a set of genes uniquely downregulated in the *in vivo* model, demonstrating the microenvironment-specific effect of maspin. GO categories closely related to interaction of cell with its environment: adhesion, migration, and wound healing are also heavily affected by maspin. Additional cancer-related processes, such as immune response and inflammation, were significantly affected *in vivo*. Previously, it was assumed that this is a consequence of the effect of secreted and membrane-bound maspin, while our current findings demonstrate that the effect of maspin on gene expression could be at least as important.

Consistent with the maspin-induced phenotypes, the cell differentiation category is overrepresented in the 3D and *in vivo* models, but not in 2D. It is worth noting that maspin seems associated with a significant enrichment in proliferation genes, especially in the 2D system, although maspin has not been reported to significantly alter the growth rate. By restoring and keeping epithelial cells in a better differentiated state, despite the microenvironment, maspin appears to curtail the plasticity or stem cell-like characteristics of tumor cells. It is intriguing to hypothesize that maspin may also play an important role in epithelial differentiation during development. Investigation of the role of maspin in development thus far is limited to the observation that maspin knockout is embryonically lethal.³³ Our analysis also shows significant change in developmental genes expression in all three model systems. Other important categories are regulation of cell death and apoptosis, consistent with the observation that maspin-induced prostate tumor cell redifferentiation was accompanied with polarized cell apoptosis,²⁶ and our earlier report that maspin sensitized tumor cells to drug-induced apoptosis.³⁴ Indeed, these categories were also enriched in all three model systems.

III. TRANSCRIPTION FACTORS REGULATED BY MASPIN

Earlier, we reported results of an extensive literature search that suggested a role of maspin in the regulation of HDAC target genes.²⁶ However, the number of those HDAC target genes appeared to be significantly smaller than that revealed when cells were treated by small-molecular weight pharmacological HDAC inhibitors. We speculate that as an endogenous HDAC inhibitor, maspin may regulate HDAC in a more specific and, thus, more selective manner, depending on the cellular and molecular contexts. To get more insights into the molecular mechanisms underlying the epigenetic effect of maspin, it is important to determine what factors mediate the maspin effects on gene transcription. In humans, there are over 2000 proteins capable to bind specific DNA sequences and influence gene transcription, allowing defining them as transcription factors (TFs).³⁵ We analyzed overrepresentation of reported and putative TF binding sites in promoter regions (–1000 to +100 from transcription start site) of differentially expressed genes relative to a cohort of 561 human housekeeping genes. Analysis of a library of more than 70,000 TF binding sites was performed using an ExPlain MATCH software tool.³⁶ The overrepresented TFs

identified by this analysis are shown in Fig. 2. To determine the core regulatory influence of maspin on gene expression, we used the lists of commonly up- and downregulated genes (the overrepresented TFs marked in red). Genes uniquely differentially expressed in 2D or *in vivo* bone tumors allowed us to track the environment-specific maspin effect (TFs marked in blue), and lists of all differentially expressed genes for every experimental condition were used to obtain the most comprehensive coverage (TFs marked in black). Statistically, the latter is the most reliable category since the number of genes analyzed for overrepresentation of TFs was over 1000 in each condition, while lists of commonly regulated genes and uniquely regulated genes consist of about 30 each and 100 each, respectively.

Similar to the GO processes, TF binding sites are overrepresented in upregulated gene subsets with lower, though still very strong, statistical significance than in downregulated subsets. This effect could be attributed to more general influence of HDACs inhibition on accessibility of DNA and thus on expression profile. On the other hand, many TFs are directly regulated by HDACs and when some of these regulatory mechanisms are well documented, there may be many others yet to be discovered. As expected, there are numerous representatives of HDAC-regulated cohort among TFs influenced by maspin. Among TFs shown in Fig. 2 that are also influenced by HDACs are Fos, Jun, E2f, Sp1, and NF- κ B. One that has to be mentioned is Zeb1, a factor responsible for HDAC1-dependent silencing of E-cadherin expression.³⁷ Consistent with this finding, we have reported that inhibition of HDAC leads to elevated E-cadherin expression in our *in vivo* experimental system.²⁶

The most abundant overrepresented TF binding sites in all conditions sets and often numerous sites per condition are those associated with Forkhead box (FOX) proteins. This finding is not surprising since members of this family participate in the transcriptional regulation of a large number of proteins that are in turn regulated by many different factors including acetylation status or acetylation-dependent interactions.^{38–40} At this time, we are unable to pinpoint the role of particular members of the FOX family in epigenetic regulation by maspin since the ExPlain tool we used in most of the cases can attribute a TF binding site to FOX protein, but not directly to a particular family member. A notable exception is Foxa, whose binding sites were significantly overrepresented in genes downregulated by maspin *in vivo*. All members of this small family of TFs were shown to be overexpressed in prostatic intraepithelial neoplasia and prostate cancer and were reported to activate the expression of androgen-dependent prostate-specific genes in an androgen receptor- and ligand-independent manner.⁴¹ In addition, Foxa1 may act as a key mediator of estrogen receptor function and endocrine response in breast cancer.⁴² Interestingly, HDAC7 has been reported to be important for Foxa1 DNA binding.⁴³ One can speculate that maspin may influence Foxa function by HDAC inhibition. Currently, it is not known whether maspin can bind and inhibit other class I HDACs such as HDAC7. It is also not clear whether HDAC7 functionally coincides with maspin under physiological or pathological conditions.

Another TF highly influenced by maspin is myocyte enhancer factor 2 (MEF2).⁴⁴ MEF2 controls expression of a variety of genes involved in cell differentiation, proliferation, morphogenesis, survival, and apoptosis of a wide range of cell types but not (thus far) in prostate.⁴⁵ Interestingly, MEF2 binding sites are overrepresented in promoters of gene that are both up- and downregulated by maspin. Others have shown that the balance between the transcription-activating functions of MEF2 induced by MAP kinase signaling and the repressive functions of class II HDACs dictates the development of many tissues.^{46,47} Numerous kinases, including protein kinase D and calcium calmodulin-dependent protein kinases, phosphorylate class II HDACs on a series of conserved serine residues. This phosphorylation promotes export of these HDACs from the nucleus to cytoplasm and the subsequent activation of MEF2.⁴⁸ The regulation of the phosphorylation of class II HDACs

thus provides a mechanism for the modulation of MEF2 target genes in response to physiological and pathological signaling. This potential mechanism would suggest that maspin may exert its influence on MEF2 by complementing the regulation of phosphorylation of class II HDACs.

In line with the observed effects of maspin on cell differentiation is the overrepresentation of Nanog, one of the key transcription factors in establishing and maintaining pluripotency of embryonic stem cells.⁴⁹ Moreover, it has been recently shown that Nanog supports stem-like characteristics of cancer cells and promotes resistance of prostate cancer to androgen deprivation.⁵⁰ The fact that Nanog is the most significantly overrepresented TF for the cohort of genes uniquely downregulated by maspin *in vivo* further confirms the importance of microenvironment in tumorigenesis and the crucial role that maspin plays in this process.

IV. REGULATORY PATHWAYS MEDIATING THE EFFECTS OF MASPIN ON GENE EXPRESSION

The large number of TFs affected by maspin expression may result from a general molecular mode of maspin action, such as by altering their acetylation state. Alternatively, maspin may exert a specific effect on cell signaling pathway that is upstream of some TFs. To determine which, if any, regulatory pathways are influenced by maspin, we used ExPain to perform upstream analysis based on TFs overrepresented in each set or combination of our experimental conditions. In our upstream analysis, the entire network of signaling interactions was utilized to suggest causative upstream key nodes that are known to modulate gene expression through the indicated TFs. The results of this analysis are shown in Fig. 3.

Based on a subset of 29 genes downregulated by maspin in all three experimental systems, we have suggested that maspin negatively regulates TGF β signaling.²⁶ The low number of influenced genes does not allow statistically significant upstream analysis, so we could not verify this conclusion. However, when using a larger subset of genes uniquely downregulated by maspin in the *in vivo* system, upstream analysis pointed to TGF β pathway as the only fit to the observed pattern of TFs [Fig. 3(a)]. Another microenvironment-specific pathway we detected was β -catenin/E-cadherin, which was the most significantly influenced in a subset of genes uniquely downregulated in 2D. This finding is in agreement with our observation that specifically in 2D system E-cadherin expression was highly downregulated.²⁶ The analysis did not find any changes in β -catenin/E-cadherin signaling in 3D or *in vivo*.

A general upstream analysis based on complete sets of differentially regulated genes in 2D, 3D, or *in vivo* revealed a number of highly interconnected pathways influenced by maspin. Upregulated genes were found to be controlled predominantly by p62 [Fig. 3(b)] and IL-1. Some network key nodes such as PKC isoforms and TRAF6 [Fig. 3(b)] are commonly shared among different experimental systems. Genes downregulated by maspin were found to be regulated by caspase 6, CDK, [Figs. 3(c) and 3(d)], Abl, caspase 8, and others. As in the case of upregulated genes, the pathways are heavily interconnected while Abl is the most abundant. Consistent with the GO and TFs analysis results, downregulated pathways are generally more statistically significant than upregulated. For example, no significant pathways were detected using the subset of genes uniquely upregulated *in vivo*.

Further experimental evidence is needed to uncover the role of maspin in regulation of these pathways. One can speculate, however, that at least in part the effect of maspin is conducted through HDAC inhibition. The role of acetylation is not only significant in switching between open and close histone-backbone of chromatin, but also in regulating the

biochemical activity and trafficking of many proteins including TFs. For example, the activity and subcellular localization of Abl can be controlled by its acetylation state.^{51,52}

V. SUMMARY

Our bioinformatic analysis based on the maspin-induced changes of gene expression profiles not only supports the biological function of maspin as a tumor suppressor, but also independently confirms the earlier functional evidence that maspin acts as an endogenous HDAC inhibitor. Since maspin is the only endogenous polypeptide HDAC inhibitor identified thus far, our data provides the first road map that connects the regulation as well as the functional consequence of fine-tuning of HDAC activity in epithelial cell biology and in epithelial response to different tissue microenvironments. Based on the biochemical activity of HDAC on a broad range of protein substrates including histones, it is reasonable to expect that HDAC inhibition by endogenous inhibitor maspin may impact a wide spectrum of genes. However, our current systematic analysis for the first time connected maspin to specific and critical signaling nodes and the transcriptional factors. Furthermore, the molecular and biological outcome of maspin-mediated HDAC inhibition is adaptive to changes of epithelial microenvironment. These results prompt us to look at maspin as an endogenous rheostat in HDAC regulation to achieve fine-tuning of epithelial homeostasis.

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FIGURE 1. Gene ontology (GO) categories affected by maspin expression. The categories are colored according to enrichment p -value (see scale).

FIGURE 2.

Transcription factors with overrepresented binding sites in promoters of genes differentially regulated by maspin. TFs based on separate analysis of each of the three experimental systems are in black, those based on lists of genes uniquely differentially expressed in specific conditions are in blue, and those based on lists of commonly regulated genes are marked in red. The TFs are listed in descending statistical significance order while only those with p -values less than e^{-5} and enrichment ratio at least 1.3 are shown.

FIGURE 3.

Network key nodes regulated by maspin according to promoter analysis. Network cluster analysis was performed using the ExPlain tool with overrepresented TFs. Activation is shown with green squares, inhibition with red, and all other interactions or modifications with yellow. TFs are shown as triangles, signaling kinases as ovals, and receptors as parallelograms.