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Re-expression and epigenetic modification of maspin induced apoptosis in MCF-7 cells mediated by myocardin

Xing-Hua Liao [a,b,1], Yan-Qi Li [a,1], Nan Wang [a,*,1], Li Zheng [a], Wen-Jing Xing [a], Dong-Wei Zhao [a], Ting-Bao Yan [a], Yue Wang [a], Long-Yue Liu [a], Xue-Guang Sun [a], Peng Hu [b], Hao Zhou [a], Tong-Cun Zhang [a,b,**]

[a] Key Laboratory of Industrial Fermentation Microbiology, Ministry of Education and Tianjin, College of Biotechnology, Tianjin University of Science and Technology, 300457, PR China
[b] Institute of Biology and Medicine, Wuhan University of Science and Technology, 430000, PR China

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ABSTRACT

Breast cancer is the leading cause of cancer death in women worldwide. It is well known that oncogene activation and anti-oncogene inactivation affect the development and progression of breast cancer, but the role of oncogene activation and anti-oncogene inactivation in breast cancer is still not fully understood. We now report that maspin acts as a tumor suppressor gene to induce MCF-7 cell apoptosis. In addition, maspin promoter hypermethylation and histone hypoacetylation lead to silencing of maspin gene expression in MCF-7 cells. Moreover, DNA methyltransferase (DNMT) inhibitor 5-aza-2′-deoxycytidine (5-aza-dc) and/or the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA) strongly up-regulated the expression of maspin in MCF-7 cells. Notably, myocardin can promote the re-expression of maspin in MCF-7 cells. Luciferase assay shows that myocardin activates the transcription of maspin promoter by CArG box. More importantly, 5-aza-dc/TSA and myocardin synergetically enhance re-expression of maspin and augment maspin-mediated apoptosis in MCF-7 cells. Thus, these data reveal the new insight that myocardin meditates apoptosis in breast cancer through affecting maspin re-expression and epigenetic modification to regulate the development of breast cancer, thereby raising the possibility of its use in breast cancer therapy.

1. Introduction

Breast cancer is one of the most common malignant tumors with high morbidity and fatality rate in females [1]. Breast cancer progression is a dynamic process controlled by multiple genetic factors, including oncogenes and tumor suppressor genes, which regulate tumor growth and progression [2]. Nowadays, it has been well proved that the formation of breast cancer is due to multi factors with oncogene activation and tumor suppressor gene inactivation [3]. Moreover, epigenetic modifications to DNA and histones serve as a tractable set of switches crucial to normal cellular differentiation and proper organism development regulating oncogene and tumor suppressor gene expression, genomic imprinting and chromosomal stability [4].

Maspin, which is a serine protease inhibitor (serpin), acts as a tumor suppressor gene and suppresses the growth and metastasis of breast tumor in vivo [5,6]. Maspin has been shown to inhibit cell motility, invasion, and metastasis, and sensitize breast cancer cells to induced apoptosis [5,7,8]. DNA hypermethylation and histone deacetylation lead to silencing of the maspin gene in human breast cancer [9,10]. However, the exact molecular mechanism regulating maspin re-expression to affect apoptosis of breast cancer cells has not been studied clearly.

Myocardin is often known as a key transcriptional regulator of cardiac and smooth muscle development [11,12]. A latest report shows that myocardin expression is markedly down-regulated in multiple types of human tumors [13]. Moreover, myocardin has also been shown to act as a tumor suppressor that forced expression of myocardin in tumor cells which can restore expression of differentiation markers and inhibit the transformed phenotype [14]. Some previous studies have suggested that the function of myocardin in differentiation defect or the acetylation of nucleosomal histones surrounding SRF-binding sites may contribute to resist the process of malignant transformation [13,15]. Thus we hypothesize that myocardin may affect the expression of tumor suppressor gene to regulate breast cancer apoptosis by epigenetic modifications.

Abbreviations: DNMT, DNA methyltransferase; 5-aza-dc, 5-aza-2′-deoxycytidine; HDAC, histone deacetylase; TSA, Trichostatin A; serpin, serine protease inhibitor; DMEM, Dulbecco's modified Eagle's medium; EB, ethidium bromide; PS, phosphatidylserine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; DAPI, 4′,6′-diamidino-2-phenylindole; ChIP, chromatin immunoprecipitation.

* Correspondence to: N. Wang, College of Biotechnology, Tianjin University of Science and Technology, Tianjin, PR China. Tel.: +86 2260 602 099; fax: +86 2260 602 298.
** Correspondence to: T.-C. Zhang, College of Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, PR China. Tel.: +86 2260 602 099; fax: +86 2260 602 298.
E-mail addresses: wn929@tust.edu.cn (N. Wang), tony@tust.edu.cn (T.-C. Zhang).
[1] These authors contributed equally to the project.

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In this study, we demonstrate that maspin acts as a tumor suppressor gene to induce apoptosis in MCF-7 cells. In addition, maspin promoter hypermethylation and histone deacetylation lead to silencing of maspin gene expression in MCF-7 cells. Moreover, DNA methyltransferase inhibitor (5-aza-dc) and/or histone deacetylase inhibitor (TSA) strongly up-regulated the expression of maspin in MCF-7 cells. Notably, myocardin has the function of promoting re-expression of maspin in MCF-7 cells. Furthermore, 5-aza-dc/TSA and myocardin synergetically enhance re-expression of maspin in MCF-7 cells. More importantly, 5-aza-dc/TSA and myocardin synergetically enhance maspin-mediated apoptosis. Thus, these data reveal the new insight that myocardin meditates apoptosis in breast cancer through affecting maspin re-expression and epigenetic modification to regulate the development of breast cancer and helps to identify new targets for therapeutic intervention.

2. Materials and methods

2.1. Cell line

MCF-7 (human breast cancer cell lines) and Hela (human cervical carcinoma cell lines) cells were obtained from American Type Culture Collection. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 incubator.

2.2. Plasmids

The pcDNA3.1-myocardin contained a cDNA encoding amino acids 1–935 of mouse myocardin and the pcDNA3.1-maspin contained a cDNA encoding amino acids 1–375 of human maspin. The vector pcDNA3.1 alone was used as a negative control. The luciferase reporter plasmids of maspin promoter (−2239 to +95) (maspin-WT-luc) including CArG box and CArG box-deleted maspin promoter (−2151 to +95) (maspin-D-luc) were cloned into the pGL3.0 vectors (Promega). The primers used were as follows: maspin-WT-luc: forward: 5′-CTGA CGCGTATACCTTGTATCATCCCAGCTCTGG-3′; reverse: 5′-ACACTCGAGG AGGAGCACAAAGACCTGGATGTG-3′ and maspin-D-luc: forward: 5′-GATACGCGTCCAGTCATAGCCAGACTGAAAAGGT-3′; reverse 5′-TAGCTC GAGGAGGAGCACAAAGACCTGGATGTG-3′.

Additional maspin promoter–reporter constructs containing mutations to putative CArG box were generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The maspin-WT-luc CArG box was changed from -CTTAA ATGG- to -CTGGATGG- (maspin-M-luc) and these nucleotide mutations abolished myocardin-SRF-binding sites. The primers used were as follows: maspin-M-luc: forward: 5′-GACCTCCGTTCTCAGGCTTGGATGGT CTCCTGATGTCCAATG-3′; reverse 5′-CATTGGACATCAGGAGACCATCCA AGCCTGAGAACGGAGGTC-3′.

2.3. MTT assay

Cell viability and proliferation were examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (Sigma). MCF-7 cells were transfected with maspin for 24 h or treated with 5-aza-dc or TSA for 5 d. Then the absorbance of each well was measured using a Synergy™ 4 plate reader (Bioteck) with a wavelength of 490 nm, with the reference wavelength set at 630 nm. Absorbance was directly proportional to the number of survival cells.

2.4. Semi-quantitative reverse transcription PCR (RT-PCR) and real-time quantitative PCR (qRT-PCR)

RT-PCR and qRT-PCR analyses were carried out as described previously [16]. RT-PCR products were visualized on 2% agarose gels stained with ethidium bromide (EB) under UV trans-illumination. qRT-PCR was performed in an Applied Biosystems StepOne™ real-time PCR system. Fast SYBR® Green Master Mix was obtained from Applied Biosystems. Data were shown as relative expression level after being normalized by glyceral-dehyde-3-phosphate dehydrogenase (GAPDH).

2.5. Western blotting

Western blotting was performed as described previously [16]. The total proteins of cells were immunoblotted with rabbit-maspin antibodies (Abcam) overnight at 4 °C and then incubated with IR Dye™-800 conjugated anti-rabbit secondary antibodies for 30 min at room temperature (RT). Specific proteins were visualized by Odyssey™ Infrared Imaging System (Gene Company). β-Actin (Santa Cruz) expression was used as an internal control to show equal loading of the protein samples.

2.6. Immunofluorescence staining

Immunocytochemistry assays were performed as described previously [17]. The cells after treatment were fixed in 4% paraformaldehyde for 15 min, and then blocked with normal goat serum for 20 min at RT. Then, rabbit anti-maspin antibodies (Abcam) were added and incubated in a humid chamber overnight. After washing with PBS thrice, cells were incubated with appropriate secondary antibodies (fluorescein isothiocyanate (FITC)-goat anti-rabbit IgG, SantaCruz) for 30 min at 37 °C. After washing with PBS, the samples were observed under a laser scanning confocal microscope (Olympus). DAPI stain (blue) highlights the total nuclei.

2.7. Luciferase reporter assay

Luciferase assays were performed as described previously [16]. After 24 h post-transfection, luciferase activity was measured on a Synergy™ 4 plate reader (Bioteck). Transfection efficiencies were normalized by total protein concentrations of each luciferase assay preparation. All experiments were performed at least three times with different preparations of plasmids and primary cells, producing qualitatively similar results.

2.8. HAT activity assay

After MCF-7 cells were transiently transfected with expression vectors encoding the myocardin, p300 and myocardin/p300 24 h later, cells were harvested and then total HAT activity was performed using HAT Activity Colorimetric Assay Kit (Upstate Biotechnology), according to the manufacturer's protocol. Mainly, samples were mixed with acetyl-CoA and incubated for 30 min on a plate precoated with histone H3 or histone H4. Acetylated histones were detected using an anti-acetyl-lysine rabbit polyclonal antibody followed by a horseradish peroxidase-based colorimetric assay.

2.9. ChIP assay

ChIP analysis was performed using a commercially available kit (Enzymatic Chromatin IP (Magnetic Beads), Cell Signaling Technology) in maspin-transfected MCF-7 cells. Proteins were cross-linked to DNA by formaldehyde at a final concentration of 1% for 20 min at room temperature. Protein–DNA complexes were immunoprecipitated using primary antibodies for myocardin (Sigma). Maspin promoter and myocardin complexes were measured by PCR. The primers used for the amplification of the human maspin promoter region between −2212 and −1943 bp were as follows: forward: 5′-GCTCTGGACCTCCGTTCT-3′, and reverse: 5′-GGGTGATTCTAACTGGGT-3′. For histone acetylation assay, protein–DNA complexes were immunoprecipitated using primary antibodies for acetyl-histone H4 (Ac-H4) (Millipore). Maspin promoter and H4 promoter complexes were measured by qPCR. The primers used for the amplification of the human maspin promoter