CHAPTER 3

RESULTS

3.1 PROTEIN EXPRESSION IN HUMAN BREAST MDA-MB-231 ADENOCARCINOMA CELLS

MDA-MB-231 cells were transfected with pcDNA3.1-6xHis-FLAG-expression vector expressing maspin wild type, ovalbumin or chimeric mutants. After 24, 48 and 72 h, protein expression of maspin, ovalbumin and chimeric mutants were analyzed by Western blotting using anti-FLAG antibodies. Maximum expression of protein at 48 h after transfection was selected for further experiments, as shown in **Figure 14**.

3.2 REQUIREMENT AND SUFFICIENCY OF MASPIN RCL FOR TUMOR SUPPRESSIVE ACTIVITY IN MDA-MB-231 CELLS

Biological function of maspin has been shown to promote breast carcinoma cell adhesion to extracellular matrix, fibronectin, and inhibit migration, invasion of the cancer cells (4). In this study, maspin RCL was determined whether this region is required and sufficient for maspin activities in MDA-MB-231 cells. Analysis of cell-fibronectin adhesion in **Figure 15** showed that maspin wild-type and OMO mutant containing maspin RCL significantly increased cell adhesion by approximately 60 percent while both ovalbumin and MOM mutant containing ovalbumin RCL were inactive. In addition, maspin and OMO markedly inhibited *in vitro* migration and Matrigel invasion by approximately 40 percent compared to mock and ovalbumin controls (**Figure 16, 17**).

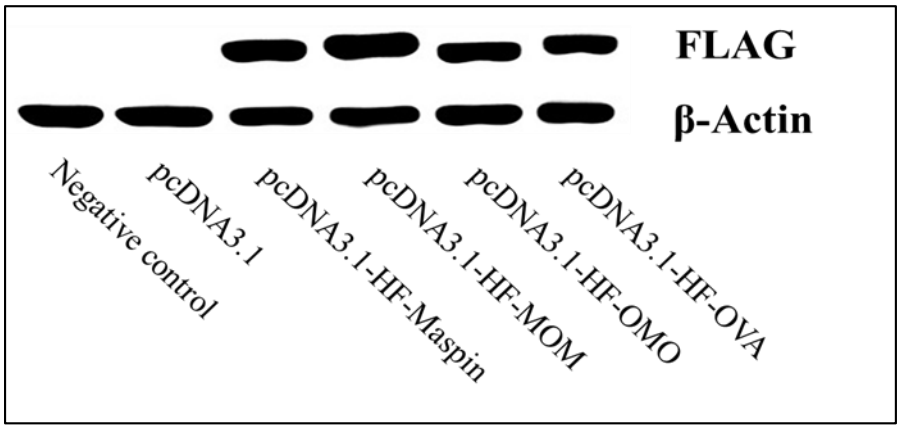

Figure 14 Ectopic Expression of Maspin, Ovalbumin and Mutants in MDA-MB-231 Cells. cDNA of Maspin, ovalbumin, and two Maspin/ovalbumin-RCL swapped mutants (MOM, OMO) containing N-terminal His/FLAG tag were transiently transfected into MDA-MB-231 cells. Non-transfected cells or transfected with empty pCDNA3.1 vector were used as negative and mock control, respectively. After 48 h transfection at 37 °C, total protein samples were extracted and subjected to Western blotting using anti-FLAG M2 antibody. β-Actin was used for normalization of gel loading.

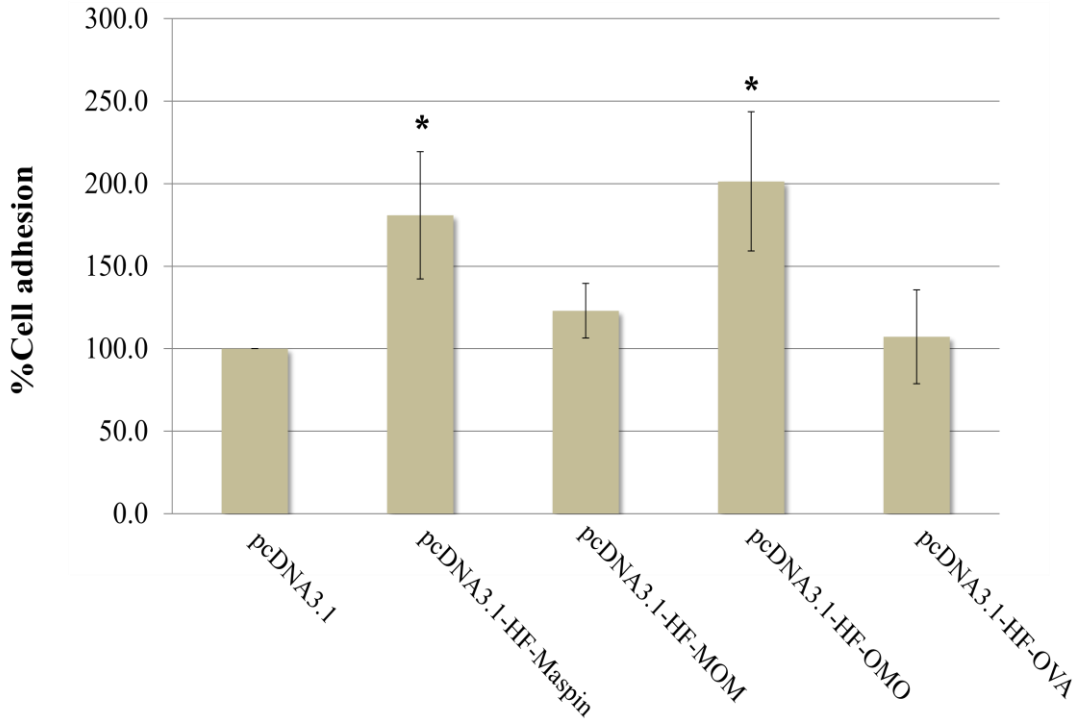

Figure 15 Requirement of Maspin RCL for Stimulation of Cell Adhesion *in vitro*. MDA-MB-231 cells were transfected with pCDNA3.1 vector alone (mock) or containing cDNA of maspin, ovalbumin, or RCL chimeric mutants (MOM, OMO). The transfectant cells ($2x10^4$) were seeded and allowed to attach for 1 h at 37°C on fibronectin-coated 96-well plate. Adherent cells were stained with 0.2% crystal violet and analyzed by measurement of absorbance at 650 nm using a microplate reader. The Y-axis shows the percentage of cell adhesion relative to the mock control. Data are mean ± SD values from three independent experiments. *$*P < 0.05$* relative to mock and ovalbumin transfected control.

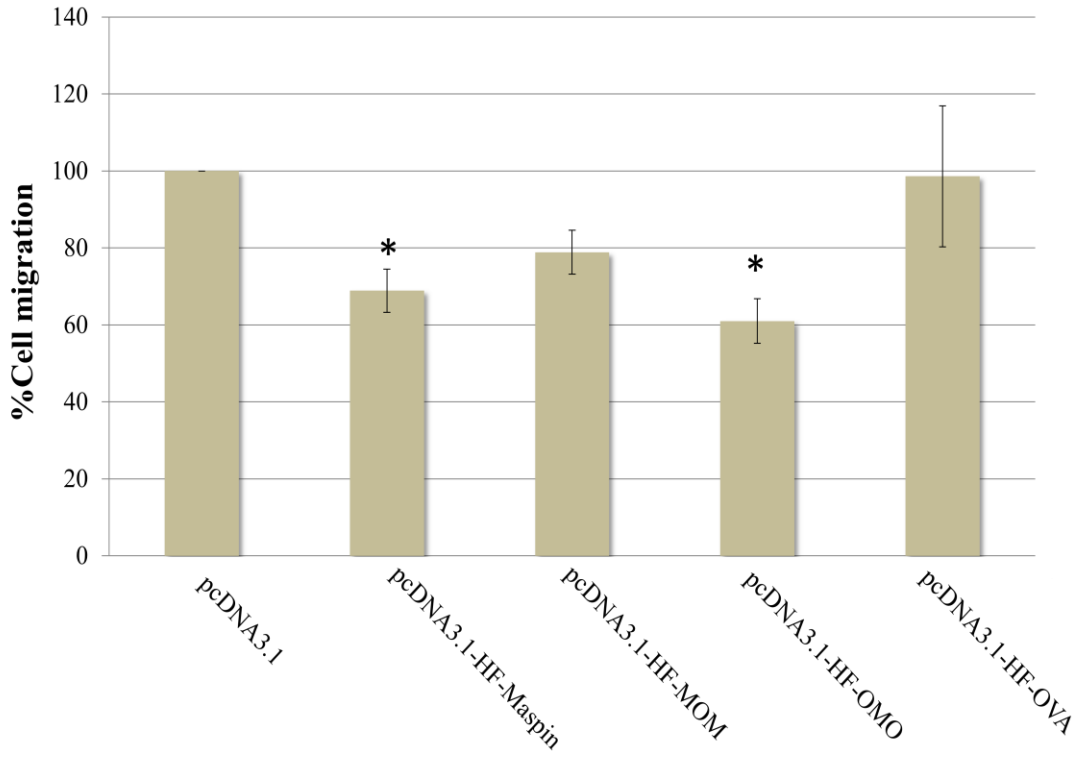

Figure 16 Requirement of Maspin RCL for inhibition of Cell Migration *in vitro*. MDA-MB-231 cells were transfected with pCDNA3.1 vector alone (mock) or containing cDNA of maspin, ovalbumin, or RCL chimeric mutants (MOM, OMO). The transfectant cells (2 x10^4) in serum free medium were added to the upper wells of the 96-well MultiScreen MIC plate. DMEM culture medium containing 10% FBS were added to lower chambers. After 24 h of incubation, the cells in the bottom well were collected and stained with fluorescent CyQuant GR dye. Fluorescence was measured using a 480/520 nm filter in a fluorometer. The Y-axis shows the percentage of cell migration relative to the mock control. Data are mean ± SD values from three independent experiments. *$P < 0.05$ relative to mock and ovalbumin transfected control.

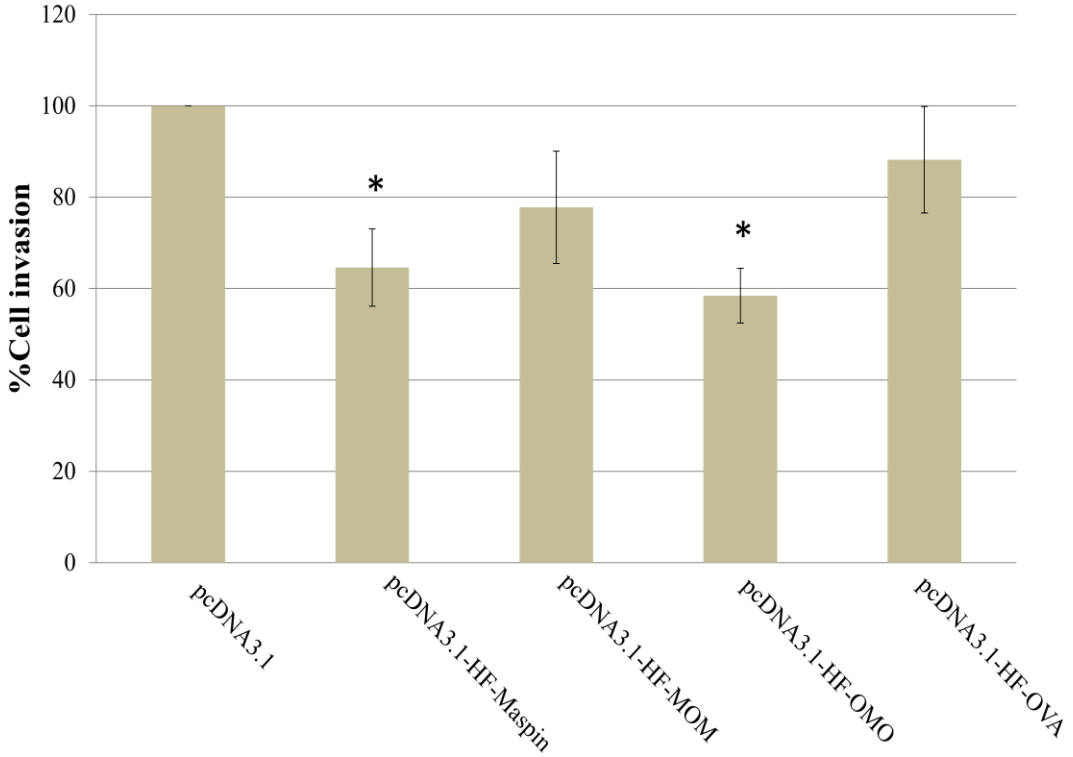

Figure 17 Requirement of Maspin RCL for Inhibition of Cell Invasion *in vitro*. MDA-MB-231 cells were transfected with pCDNA3.1 vector alone (mock) or containing cDNA of maspin, ovalbumin, or RCL chimeric mutants (MOM, OMO). *In vitro* Matrigel invasion assay was performed in Multiscreen MIC 96-well plate format. The transfectant cells (2 x10^4) in serum free medium were added to the upper wells in which Matrigel was pre-coated. DMEM culture medium containing 10% FBS were added to lower chambers. After 48 h of incubation, the cells in the bottom well were collected and stained with fluorescent CyQuant GR dye. Fluorescence was measured using a 480/520 nm filter in a fluorometer. The Y-axis shows the percentage of cell invasion relative to the mock control. Data are mean ± SD values from three independent experiments. *$P < 0.05$ relative to mock and ovalbumin transfected control.

3.3 THE RCL OF MASPIN IS NECESSARY FOR INHIBITION OF CHYMOTRYPSIN-LIKE ACTIVITY OF THE 20S PROTEASOME.

Previous study has demonstrated the effect of maspin expression on the chymotrysin-like activity of the 20S proteasome (9). Re-expression of maspin in MDA-MB-435 cells reduced chymotrypsin-like activity of 20S proteasome and led to the accumulating of the high molecular weight ubiquitin conjugates in maspin-transfected cells. These results suggest an inverse correlation between the expression of maspin and the proteasome activity. However, whether maspin RCL is required for this activity of maspin has not been shown. In this study, maspin and mutant transfectant MDA-MB-231 cells were analyzed for the accumulation of high molecular weight ubiquitin conjugates by immunoblot using anti-ubiquitin antibodies. The result is shown in **Figure 18** that high molecular weight ubiquitin-protein conjugates was noticeably accumulated in maspin and OMO transfected cells compared with control, and cells transfected with MOM or ovalbumin. Next, 20S proteasome activity of the cell lysate was determined as shown in **Figure 19**. Proteasome chymotrypsin-like activity was significantly reduced by approximately 20 percent in maspin and OMO transfected cells compared to mock and ovalbumin transfected controls.

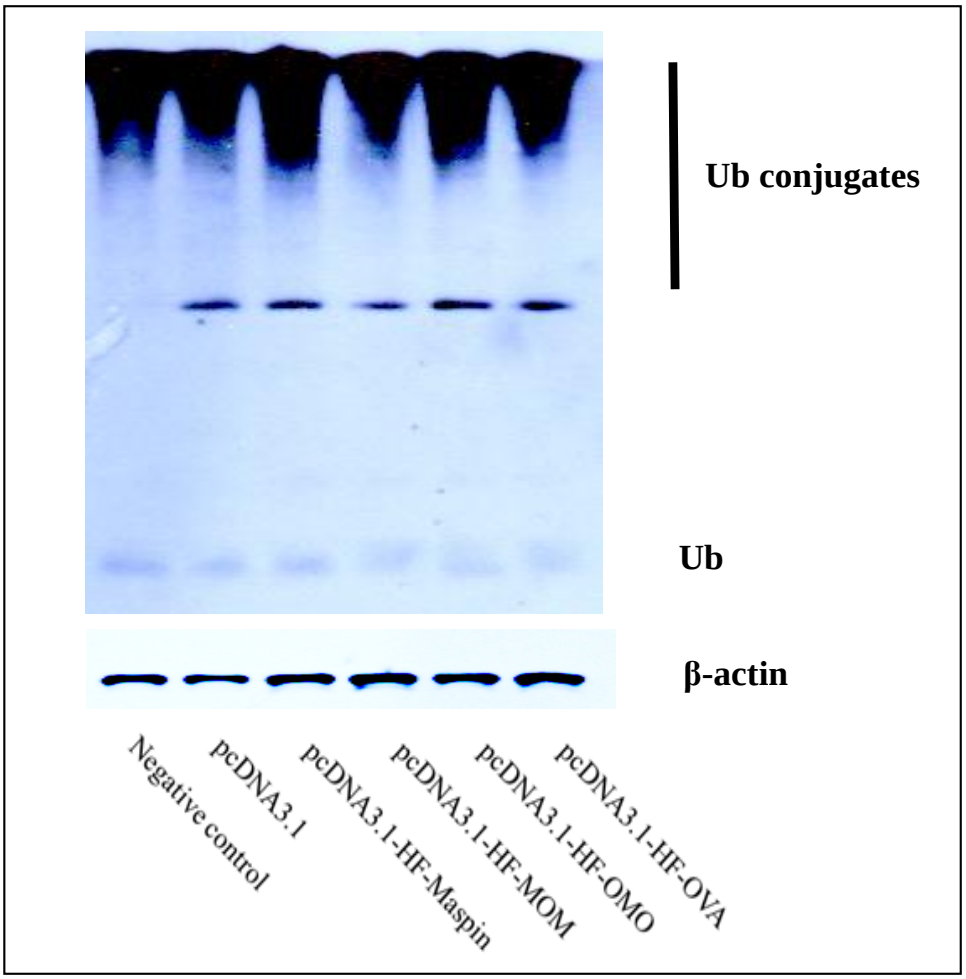

Figure 18 Effect of Accumulation of Ubiquitin-conjugates in MDA-MB-231 Cells Expressing Maspin, Ovalbumin or RCL Chimeric Mutants (MOM, OMO) transgene. The cells were transfected with pcDNA3.1-6xHis-FLAG expression vectors containing wild type maspin, ovalbumin, or chimeric RCL mutants MDA-MB-231 cells. Transfectant cells were harvested after 48 h of transfection at 37 °C. Total protein samples were extracted and subjected to immunoblotting using polyclonal anti-ubiquitin antibodies. β-actin was used for normalization.

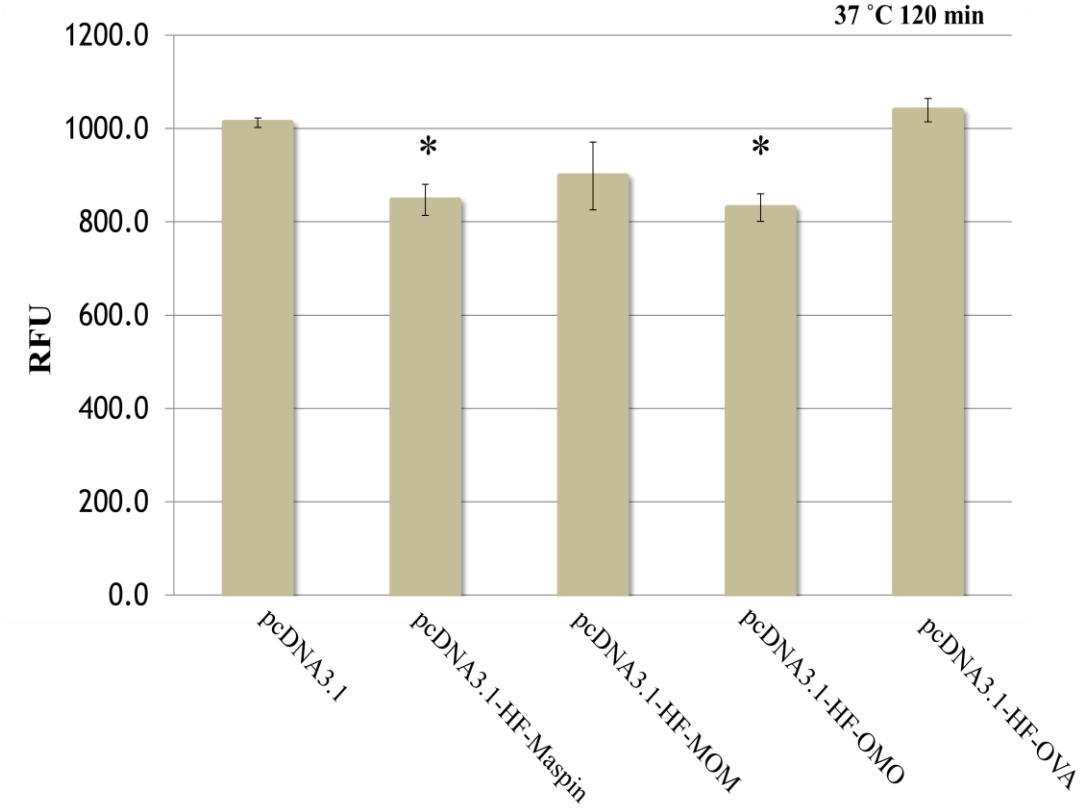

Figure 19 Effect of Ubiquitin-proteasome Activity in MDA-MB-231 Cells Expressing Maspin, Ovalbumin or RCL Chimeric Mutants (MOM, OMO) Transgene. The transfectant cells were harvested and homogenized in ice-cold lysis buffer without protease inhibitors. Cellular chymotrypsin-like activity of 20S proteasome was assayed in a 96-well plate using the fluorogenic peptide substrate LLVY-AMC and measured using a 380/460 nm filter in a fluorometer. (RFU: relative fluorescent unit). The Y-axis shows the percentage of cell invasion relative to the mock control. Data are mean ± SD values from three independent experiments. *P < 0.05 relative to mock and ovalbumin transfected control.

3.4 EFFECT OF MASPIN RCL ON THE PROTEOME OF MDA-MB-231 CELLS

Re-expression of maspin in MDA-MB-435 cells leads to alternation in the expression level of proteome associated with actin cytoskeleton, apoptosis and ubiquitin-proteasome pathway (9). In order to investigate the effect of the maspin RCL on the proteome of MDA-MB-231 cells, total cell lysate of the transfectant cells was initially analyzed by 2-D gel electrophoresis. The results of 2-D gel electrophoresis were shown in **Figure 20**. Overall pattern of protein spots was similarly observed in all 2-D gels of the transfectant lysates. Next, image analysis of 2-D gel electrophoresis was able to detect one spot of protein that was present only in the maspin and OMO transfectants. This protein has pI ~ 4.1 and M.W ~40 kDa (**Figure 21**).

To obtain data of proteins differentially expressed among transfectants, the total cell lysates of each tansfectant were further analyzed using a highly sensitive technique called shotgun proteomic. GeLC-MS/MS analysis identified a total of 3,122 proteins. The levels of several proteins (viz. adenylosuccinate lyase, G1 phase-specific gene, glutamate carboxypeptidase) were similar between mock and maspin transfected cells, but highly different from negative control, indicating alterations of protein expression due to transfection procedure. Among the 68 proteins significantly differentially expressed, there were 31 proteins up-regulated and 37 proteins down-regulated in cells expressing maspin and OMO compared to negative, mock, MOM and ovalbumin transfected cells (**see Appendix D**). As listed in **Table 3**, examples of proteins up-regulated > 2-fold include protocadherin gamma-A5 isoform 2 precursor

(gi|14196471), alpha 1 chain-like collagen COLA1L precursor (gi|17974510), connexin45 (gi|424134) and sorting nexin-33 (gi|23397574). Those down-regulated included proteins involved in tumor cell growth, invasion and progression (viz. Interleukin 17 receptor E (gi|21756363), epithelial cell transforming 2 (gi|34978932), Rho GDP-dissociation inhibitor 2 (gi|56676393), and metallopeptidase (gi|4138016)); and proteins involved in ubiquitin-proteasome pathway, including proteasome subunit alpha type-1 (gi|4506179) and beta type-3 (gi|22538465).

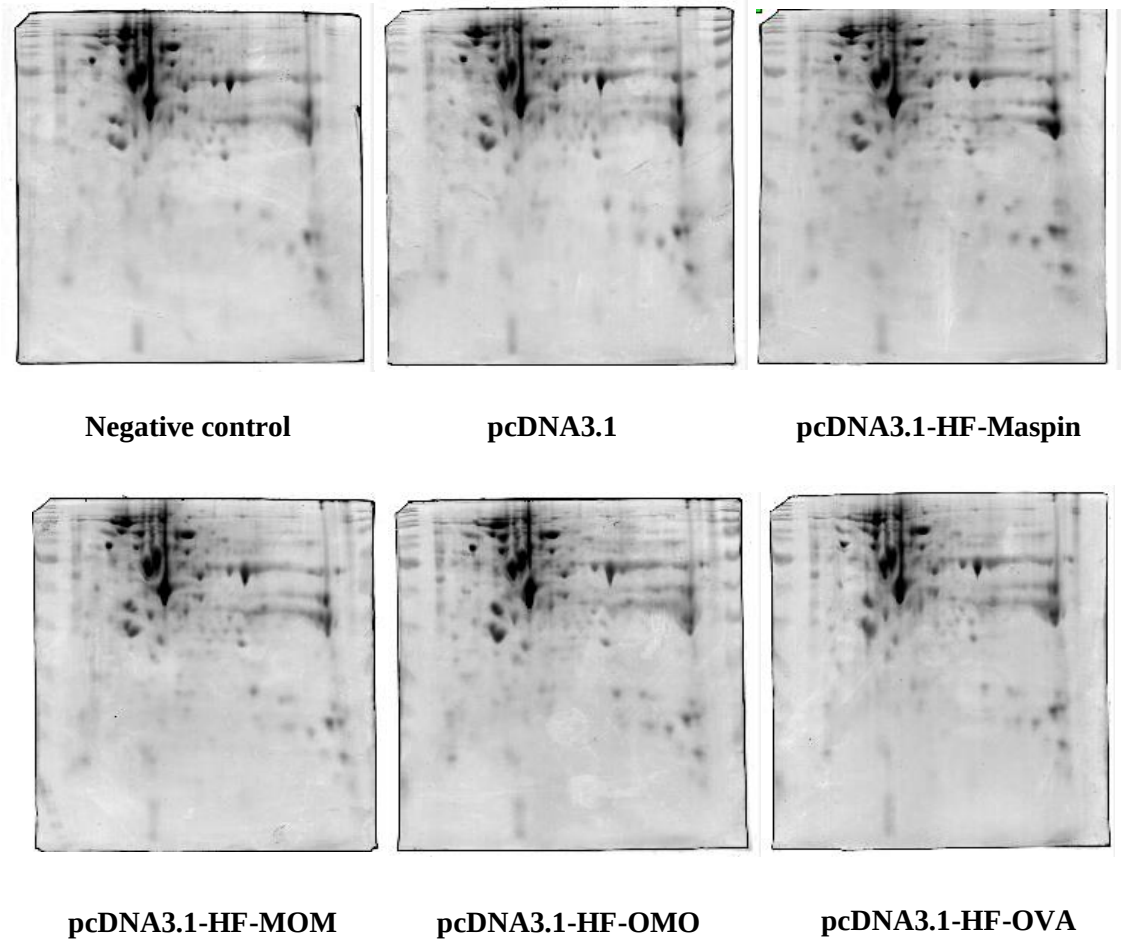

Figure 20 Effect of the Maspin RCL on the Proteome of MDA-MB-231 Cells Analyzed by 2D-gel Electrophoresis. Total cell lysate of the MBA-MB-231transfectants was separated by their charges and sizes in 2D-gel electrophoresis. Spots of protein were visualized by staining with Coomassie Brilliant Blue G250.

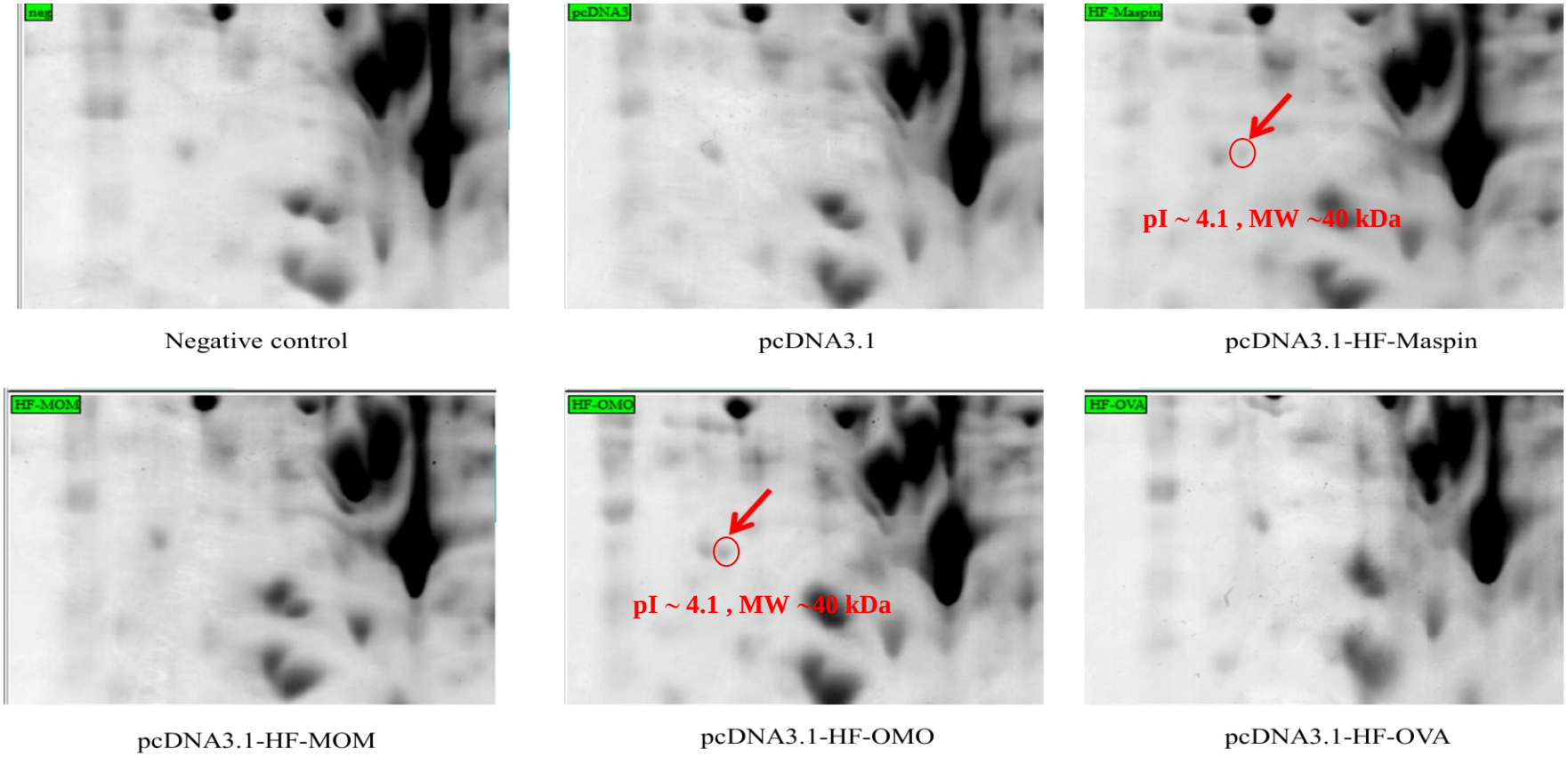

Figure 21 Differential Expression of Protein in MDA-MB-231 Cells by the Maspin RCL.

The protein expression profiles of the 2-D gels were identified by the image analysis software (ImageMaster 2D Platinum 7)

Table 3 List of Example Proteins Significantly Differentially Expressed in MDA-MB-231 Cells by the RCL of Maspin

Protein name	Accession number	MOWSE Score	Neg mean	Mock mean	Maspin mean	MOM mean	OMO mean	Oval mean
Protocadherin gamma-A5 isoform 2 precursor	gi|14196471	13.53	0.00	0.00	7.67	0.00	5.81	3.69
Alpha 1 chain-like collagen COLA1L precursor	gi|17974510	7.68	1.22	0.00	5.52	1.78	4.31	2.68
SH2 domain-containing protein 4B isoform 2	gi|224591408	14.05	2.26	0.00	5.24	0.00	7.52	2.42
Small ubiquitin-related modifier 3 precursor	gi|48928058	63.89	3.23	1.76	5.62	2.37	6.24	2.93
Connexin45	gi|424134	15.5	2.26	1.38	4.23	0.00	3.74	1.93
Sorting nexin-33	gi|23397574	30.28	2.45	2.01	6.06	0.00	6.47	1.90
Proteasome subunit beta type-3	gi|22538465	61.11	7.18	5.32	4.72	5.09	0.00	6.73
Proteasome subunit alpha type-1 isoform 2	gi|4506179	15.08	4.67	2.27	1.61	5.68	1.57	4.58
Metallopeptidase	gi|4138016	10.16	5.94	4.09	2.88	4.66	2.88	4.93
Rho GDP-dissociation inhibitor 2	gi|56676393	9.62	4.32	4.38	1.99	4.12	1.92	4.14
Epithelial cell transforming 2	gi|34978932	12.55	5.27	8.08	2.60	7.55	2.59	5.13

3.5 VALIDATION OF MASPIN RCL-REGULATED GENE EXPRESSION BY RT-qPCR

To further confirm the effect of maspin RCL on expression of target genes identified by shotgun proteomics, the levels of target mRNA were quantitatively analyzed by reverse transcription-polymerase chain reaction (RT-qPCR) method. Four down-regulated genes were chosen as their functions correlate with the activity of maspin. Epithelial cell transforming 2 and Rho GDP-dissociation inhibitor 2 are involved in cell migration, invasion whereas proteaosme subunit beta type-3, and alpha type-1 are associated with and ubiquitin-proteasome pathway. The results of RT-qPCR showed in **Figure 22, 23** that re-expression of maspin and OMO mutant MDA-MB-231 cells caused significant decreases in the transcript levels of both epithelial cell transforming 2 and proteasome subunit beta type-3. Although the mRNA levels of Rho GDP-dissociation inhibitor 2 and proteasome subunit alpha type-1 seem to decrease in the carcinoma cells expressing maspin wild-type relative to mock control, no significant difference was detected among transfectants of maspin, ovalbumin and RCL chimeric mutants **(Figure 24, 25)**.

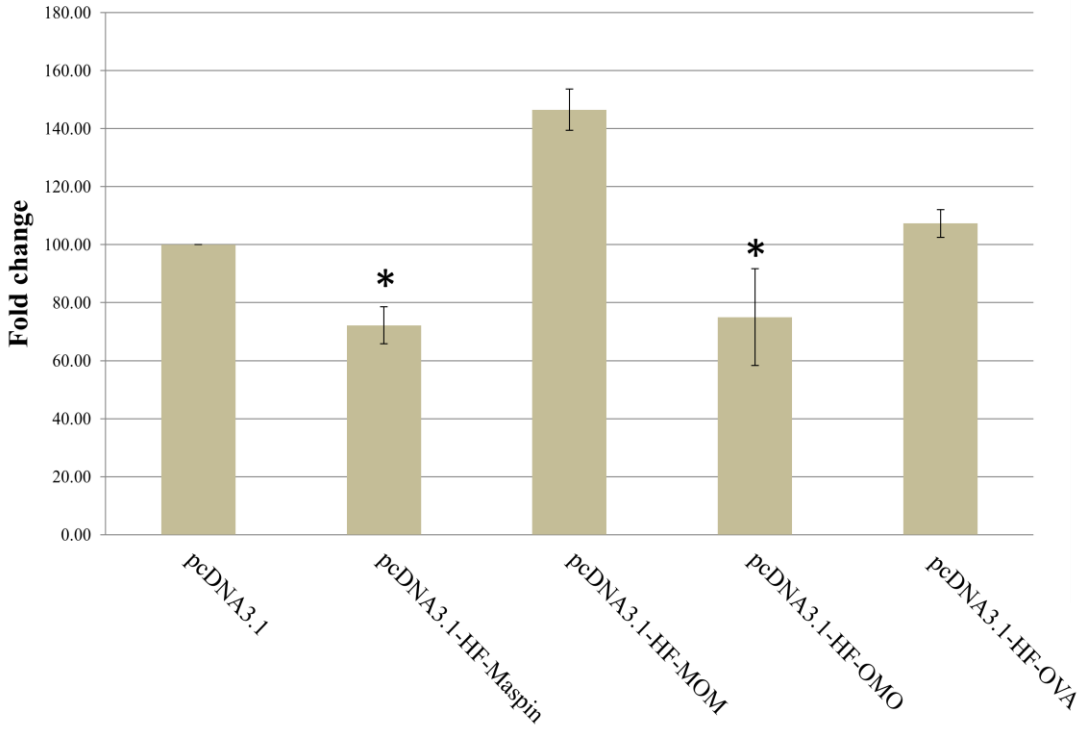

Figure 22 Down-regulation of Epithelial Cell Transforming 2 Expression by the Maspin RCL in MDA-MB-231 Cells. Total mRNA of MDA-MB-231 transfectants were extracted using Trizol method and reverse-transcribed into cDNA. qPCR reactions were performed in an ABI 7500 Real-time PCR system (Applied Biosystems, USA) using Maxima^{TM} SYBR Green qPCR Master Mix (Fermentas, Germany). An automated melting curve analysis was used to verify that all primers yielded a single PCR product. Relative expression levels for targets genes were normalized to the expression of GAPDH by the $2^{-\Delta CT}$ method. The Y-axis shows the percentage of fold change relative to the mock control. Data are mean ± SD values from three independent experiments. *P < 0.05 relative to mock and ovalbumin transfected control.

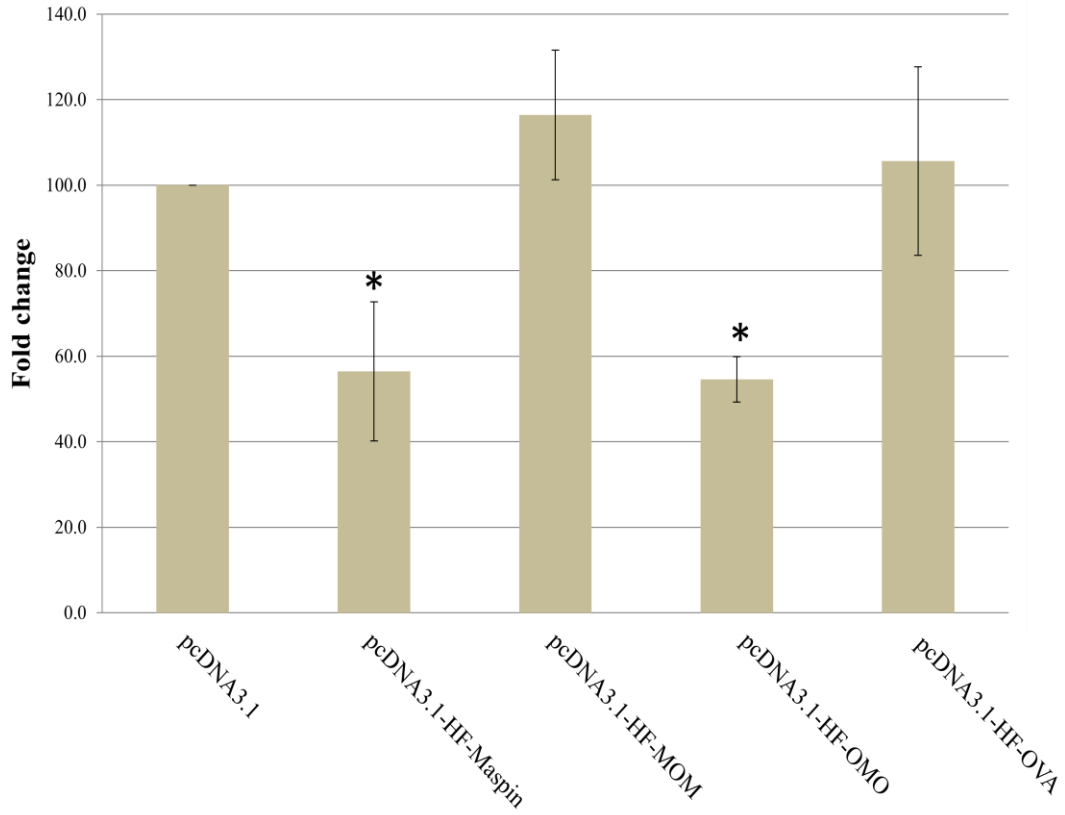

Figure 23 Down-regulation of Proteasome Subunit Beta Type-3 Expression by the Maspin RCL in MDA-MB-231 Cells. Total mRNA of MDA-MB-231 transfectants were extracted using Trizol method and reverse-transcribed into cDNA. qPCR reactions were performed in an ABI 7500 Real-time PCR system (Applied Biosystems, USA) using Maxima^{TM} SYBR Green qPCR Master Mix (Fermentas, Germany). An automated melting curve analysis was used to verify that all primers yielded a single PCR product. Relative expression levels for targets genes were normalized to the expression of GAPDH by the $2^{-\Delta CT}$ method. The Y-axis shows the percentage of fold change relative to the mock control. Data are mean ± SD values from three independent experiments. *P< 0.05 relative to mock and ovalbumin transfected control

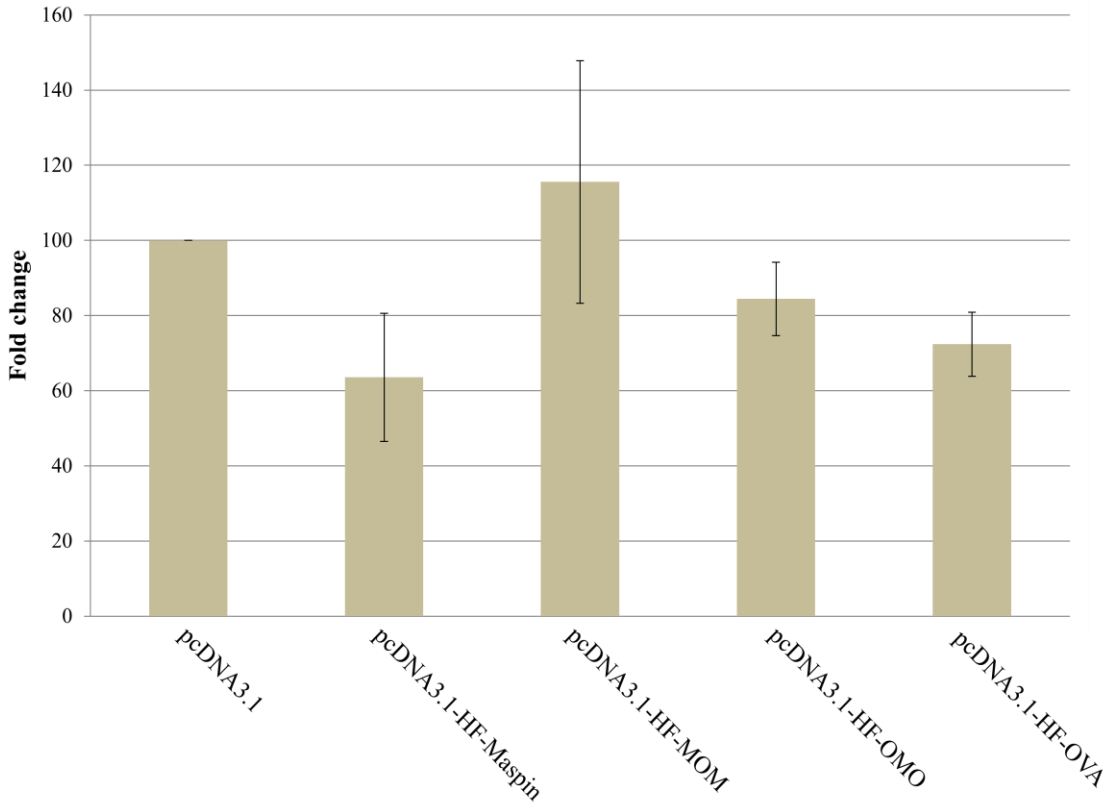

Figure 24 Expression of Rho GDP-dissociation Inhibitor 2 in MDA-MB-231 Cells Expressing Maspin, Ovalbumin or RCL Chimeric Mutants (MOM, OMO) Transgene. Total mRNA from MDA-MB-231 transfectants were extracted using Trizol method and reverse-transcribed into cDNA. qPCR reactions were performed in an ABI 7500 Real-time PCR system (Applied Biosystems, USA) using Maxima^{TM} SYBR Green qPCR Master Mix (Fermentas, Germany). An automated melting curve analysis was used to verify that all primers yielded a single PCR product. Relative expression levels for targets genes were normalized to the expression of GAPDH by the $2^{-\Delta CT}$ method. The Y-axis shows the percentage of fold change relative to the mock control. Data are mean ± SD values from three independent experiments.

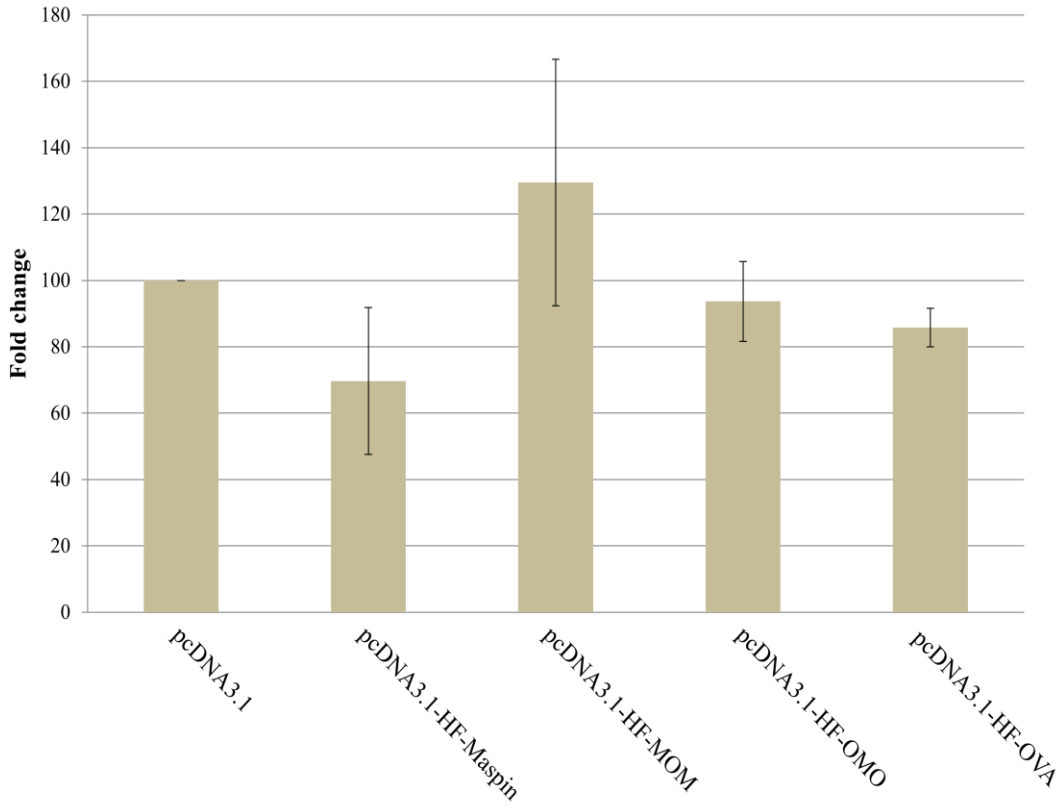

Figure 25 Expression of Proteasome Subunit Alpha Type-1 in MDA-MB-231 Cells Expressing Maspin, Ovalbumin or RCL Chimeric Mutants (MOM, OMO) Transgene. Total mRNA of MDA-MB-231 transfectants were extracted using Trizol method and reverse-transcribed into cDNA. qPCR reactions were performed in an ABI 7500 Real-time PCR system (Applied Biosystems, USA) using Maxima^{TM} SYBR Green qPCR Master Mix (Fermentas, Germany). An automated melting curve analysis was used to verify that all primers yielded a single PCR product. Relative expression levels for targets genes were normalized to the expression of GAPDH by the $2^{-\Delta CT}$ method. The Y-axis shows the percentage of fold change relative to the mock control. Data are mean ± SD values from three independent experiments.