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Inhibitory effect of obovatal on the migration and invasion of HT1080 cells via the inhibition of MMP-2

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Abstract—Because the activation of matrix metalloproteinases (MMP) is a key factor in the metastatic process, compounds with the ability to inhibit MMP activity have a potential in the treatment of tumor. From the examination of 2000 plant extracts, obovatal isolated from the extract of the leaves of *Magnolia obovata* THUNB was a potent inhibitor of MMP-2 enzyme in vitro. In human fibrosarcoma cells (HT1080) activated with MMP-2, obovatal inhibited MMP-2 enzyme activity and expression. In addition, the compound blocked migration and invasion of the cells. This study demonstrates that obovatal exerts its anticancer effects through blocking migration and invasion by inhibition of MMP-2 expression and activity, and also will be a good lead molecule for the development of anti-tumor drug.

1. Introduction

Matrix metalloproteinases (MMPs) and zinc-dependent proteolytic enzymes play an important role in matrix degradation during the tumor growth, invasion, and tumor-induced angiogenesis.[1] Especially MMP-2 can degrade type IV collagen, one of the major components of the basement membrane, resulting in the promotion of tumor invasion and metastasis.[2] When the enzyme activity of MMP-2 is blocked, their invasion activity is abolished. In the past few years, a large number of small molecules were developed as inhibitors of MMPs. Matrix metalloproteinase inhibitors (MMPIs) such as batimastat and marimastat have demonstrated their ability to delay primary tumor growth and to block metastasis.[3,4] And it was reported that various flavonoids such as chatechin, quercetin, and phorolentin inhibited metastatic activities by reducing MMP production of tumor cells. [5]

Natural products contain a variety of chemo-preventive compounds that have been shown to prevent the development of malignancies.[6,7] In continued screening of plant extracts for potential anti-tumor compounds,[8,9] we have tested 2000 plant extracts including 150 herbal medicines against MMP-2. We selected the extract of the leaves of *Magnolia obovata* THUNB as a good candidate for isolation of MMP-2 inhibitor. *Magnolia obovata* THUNB has been used as herbal medicine and neolignans such as magnolol, honokiol, and obovatol, isolated from that, have been well investigated. These compounds have been known to have many biological activities such as anti-platelet aggregation,[10] cholesterol acyltransferase inhibition,[11] and anti-inflammatory action.[12] Especially, magnolol and honokiol showed anti-tumor effects through inhibition of tumor growth,[13] tumor cell invasion,[14] metastasis,[15] angiogenesis,[13] and induction of apoptosis.[16,17] Although obovatal is a compound isolated from the leaves of *M. obovata* THUNB,[18] there is no study about its biological activity on cancer.

In the present study, we described the activity-guided isolation from the leaves of *M. obovata* and its inhibitory activity on MMP-2. We further investigated to evaluate effects of obovatal on human fibrosarcoma cells (HT1080) activated MMP-2. Our data have suggested for the first time that obovatal exerts its anticancer effects through blocking migration and invasion by inhibition of MMP-2 expression and activation.

Keywords: Matrix metalloproteinase-2 (MMP-2); Obovatal; *Magnolia obovata* THUNB; Cell migration; Cell invasion.
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2. Results and discussion

2.1. Isolation and MMP-2 enzyme inhibition activity of obovatal

We screened 2000 plant extracts including 150 herbal medicines against MMP-2 enzyme assay to find a good and selective modulator of MMP-2 activity. Extract of the *M. obovata* THUNB was selected as a good natural source for MMP-2 enzyme inhibitor and the major active fraction was collected and chromatographed over silica gel column. And the combined active fractions were rechromatographed on silica gel column and then C-18 column. Structure of the isolated compound was elucidated by spectroscopic method including mass spectral and NMR data, and identified by comparison with the reported spectral data as known obovatal (Fig. 1).[18] Obovatal inhibited the MMP-2 enzyme activity in a dose-dependent manner. As shown in Figure 2, obovatal inhibited MMP-2 activity with an IC_{50} value of 0.8 ± 0.1 μM.

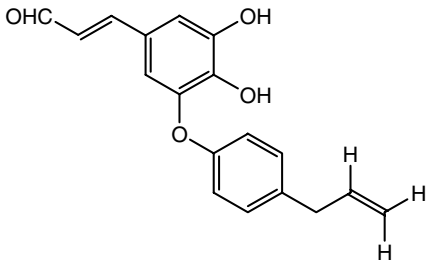

Figure 1. Structure of obovatal.

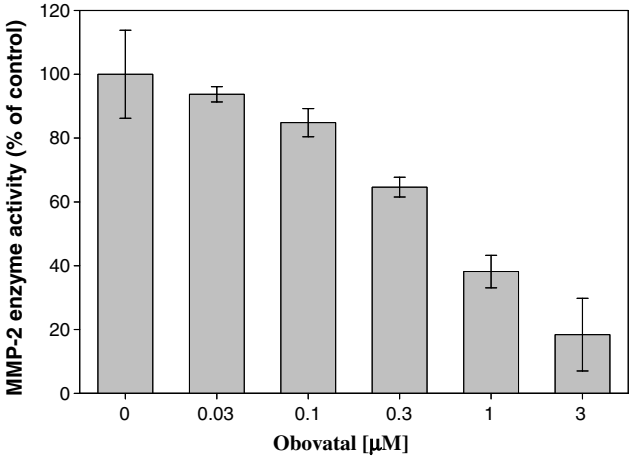

Figure 2. Effect of obovatal on MMP-2 enzyme activity in vitro. Purified recombinant MMP-2 and fluorescent substrate were used. Obovatal was added to wells of microtiter plates, each of which contained 100 μl of TNC buffer. The enzyme stock solution (5 ng/ml) was added to this solution and preincubated at 37 °C for 10 min. Then, 50 μl of substrate sol (10 μM) was added to the mixture to begin the reaction. The fluorescence values were measured at an excitation wavelength of 328 nm and an emission wavelength of 393 nm after incubation at 37 °C for 1 h. The error bars represent the standard deviation. Data are representative of three independent experiments.

2.2. MMP-2 activity and expression reduced by obovatal

To clarify whether obovatal could inhibit MMP-2 enzyme activity in HT1080 cells, gelatin zymography assay was performed under a serum starvation condition with/without obovatal addition. As shown in Figure 3a, MMP-2 enzyme activity was significantly reduced in the conditioned medium treated with 15 μM obovatal. MMP proteolytic activity is regulated at three main levels—transcription, proenzyme activation, and inhibition.[19] In order to determine whether the obovatal would reduce transcription and protein levels of

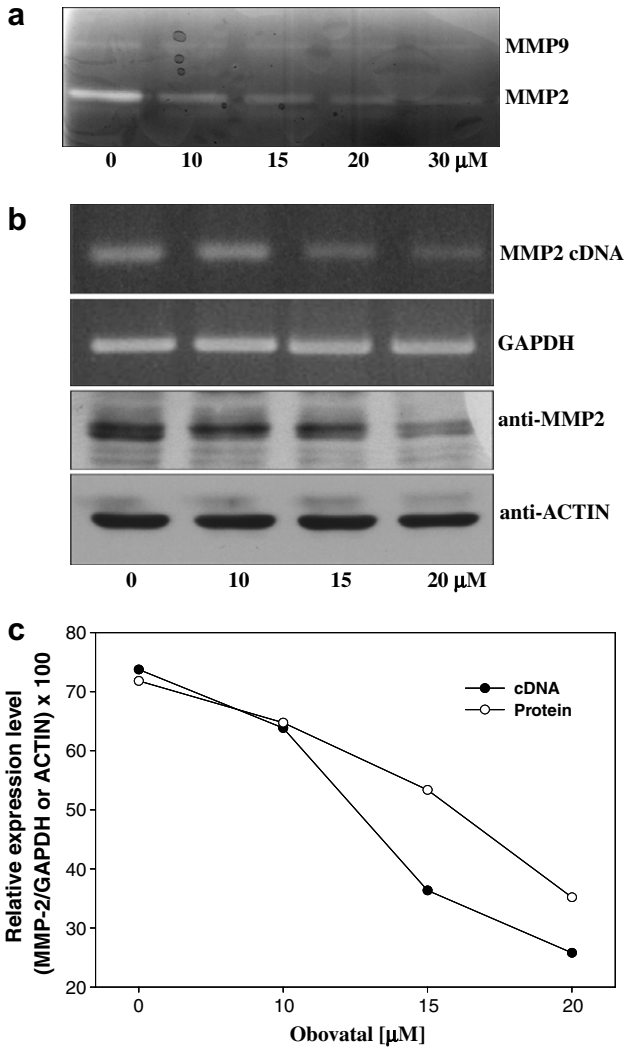

Figure 3. Effect of obovatal on MMP-2 activity and expression in HT1080 cells. (a) Zymogram analysis of gelatinolytic activity. Cells were cultured 24 h in RPMI1640 (10% FBS) containing obovatal. Cells were cultured for an additional 12 h in serum-free medium containing obovatal before collection of the conditioned medium for MMP assays. Proteins in the conditioned media were separated with 10% SDS–PAGE containing gelatin. The unstained bands corresponded to the areas of collagen digestion. (b) RT-PCR and Western blot analysis were performed as described in Section 4. The cells were treated with either vehicle or obovatal for 24 h. (c) Band intensity was quantified by Quantity ONE software (Bio-Rad). The relative expression levels indicated the MMP-2/GAPDH (cDNA) or MMP-2/ACTIN (protein) ratios. A typical result from a single experiment is shown.

MMP-2, RT-PCR and Western blotting were performed. As shown in Figure 3b, the mRNA level and protein level were reduced by treatment of obovatal. In HT1080 cells treated with obovatal (20 μM), the MMP-2 expressions were reduced by more than 70% compared with the expression in the vehicle; 0.1% DMSO (Fig. 3c).

2.3. Obovatal inhibited migration and invasion of HT1080 cells

To understand biological activity of obovatal on cancer cells, we investigated whether the obovatal is able to inhibit growth of the various tumor cells with the cell proliferation assay. Obovatal did not significantly inhibit the growth of human tumor cells up to 50 μM except HCT116 cell line (Fig. 4). Especially, it did not affect the growth of HFF (human foreskin fibroblast) cells, which means that it might be not toxic to normal fibroblast cell line. To the best of our knowledge, this is the first report of a cell viability of obovatal against normal and human tumor cells.

Because MMP-2 has been reported to have major role during the migration and invasion processes of cancer cell,[20,21] it is necessary to determine whether obovatal inhibits the invasiveness and motility of tumor cells. For confirming the activity in cells, wound healing assay and Matrigel invasion assay were also performed using HT1080 human fibrosarcoma cells. As shown in Figure 5, obovatal significantly inhibited invasion and motility of HT1080 cells at a 20 μM concentration. The quantitative analysis showed that the compound reduced the cell invasion by more than 70% compared with that of the vehicle. The results mean that obovatal inhibits the tumor cell invasion and migration without cytotoxicity.

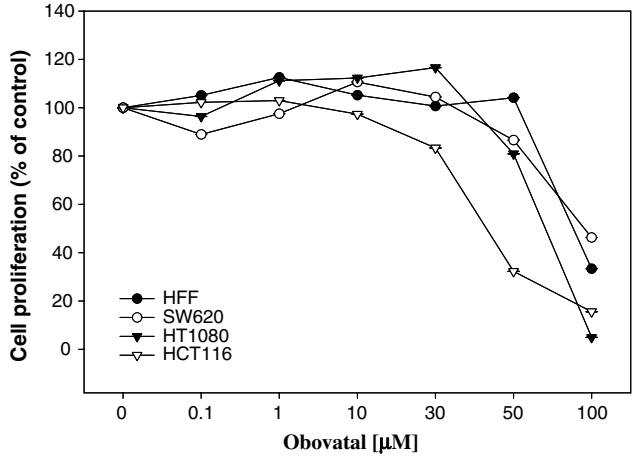

Figure 4. Effect of obovatal on the cell growth in normal and tumor cells. Various concentrations of obovatal were added to 5×10^3 cells suspended in the culture medium supplemented with 10% FBS, in a 96-well microtiter plate. Viable cells were measured by cell proliferation reagent WST-1 (Roche) after incubating at 37 °C for 48 h in a 5% CO_2 atmosphere. The amount of WST-1-formazan produced was measured at 450 nm by ELISA Reader (Bio-Rad). The error bars (smaller than the symbols) represent the standard deviation from the triplicate data set.

3. Conclusion

Obovatal was a compound isolated from the leaves of *M. obovata* THUNB, however, no one reported its biological activity in cells. In the present study, we found that obovatal could inhibit enzyme activity and expression of MMP-2. Furthermore, we confirmed that obovatal significantly inhibited motility and invasion of HT1080 cells at non-toxic concentration. Although, further investigations will be needed, obovatal might offer a therapeutic basis for preventing cancer metastasis and could be a good lead molecule for the development of anti-tumor agents.

4. Experimental

4.1. General

Chemicals and solvents were of reagent grade and used without further purification. The column fractions were monitored by thin layer chromatography (TLC) on precoated Merck Silica gel $60F_{254}$ plates; the spots were visualized by exposure to UV radiation. ^{1}H NMR was recorded on a Varian 400 MHz spectrometer in $CDCl_3$. Column chromatography (CC) separations were carried out by using silica gel 60 (0.04–0.063 mm) and LiChroprep RP-18 (40–63 μm) supplied by E. Merck.

4.2. Plant material

The leaves of *M. obovata* THUNB were collected on July 2006 in Taejeon, Korea. The authenticity of the plant was confirmed by Plant Diversity Research Center and a voucher specimen for *M. obovata* THUNB is deposited in Korea Research Institute of Bioscience & Biotechnology, Korea. The samples were dried and milled to powder form.

4.3. Isolation of obovatal

The dried leaves (5.3 kg) of *M. obovata* THUNB were extracted with MeOH for 48 h at room temperature. After the combined extract was filter and concentrated, the residue was partitioned between H_2O and EtOAc (1:1, v/v) to give EtOAc-soluble fraction (35.2 g). The EtOAc fraction showed MMP-2 inhibition activity and the EtOAc soluble fraction was concentrated, and then the residue was chromatographed on a silica gel (2 kg) column, eluted with a gradient of *n*-hexane-EtOAc to provide 10 fractions. Active fractions were collected and concentrated to yield 110 mg. The active fraction was re-subjected to a C18 column and it was eluted with a gradient of MeOH-H_2O (6:4, 7:3, 8:2, MeOH, each about 3 L) to provide obovatal (12 mg).[18]

^{1}H NMR ($CDCl_3$): δ 9.35 (H-9, d, J = 7.5 Hz), 7.38 (H-7, d, J = 15.3 Hz), 7.07 (H-3′ and H-5′, d, J = 9 Hz), 6.98 (H-4, d, J = 1.8 Hz), 6.78 (H-2′ and 6′, d, J = 9 Hz), 6.75 (H-6, d, J = 1.8 Hz), 6.42 (H-8, dd, J = 7.5, 15.3 Hz), 4.51 (H-8′, m), 5.09 (H-9′, m), 3.30 (H-7′, d, J = 6.6 Hz).

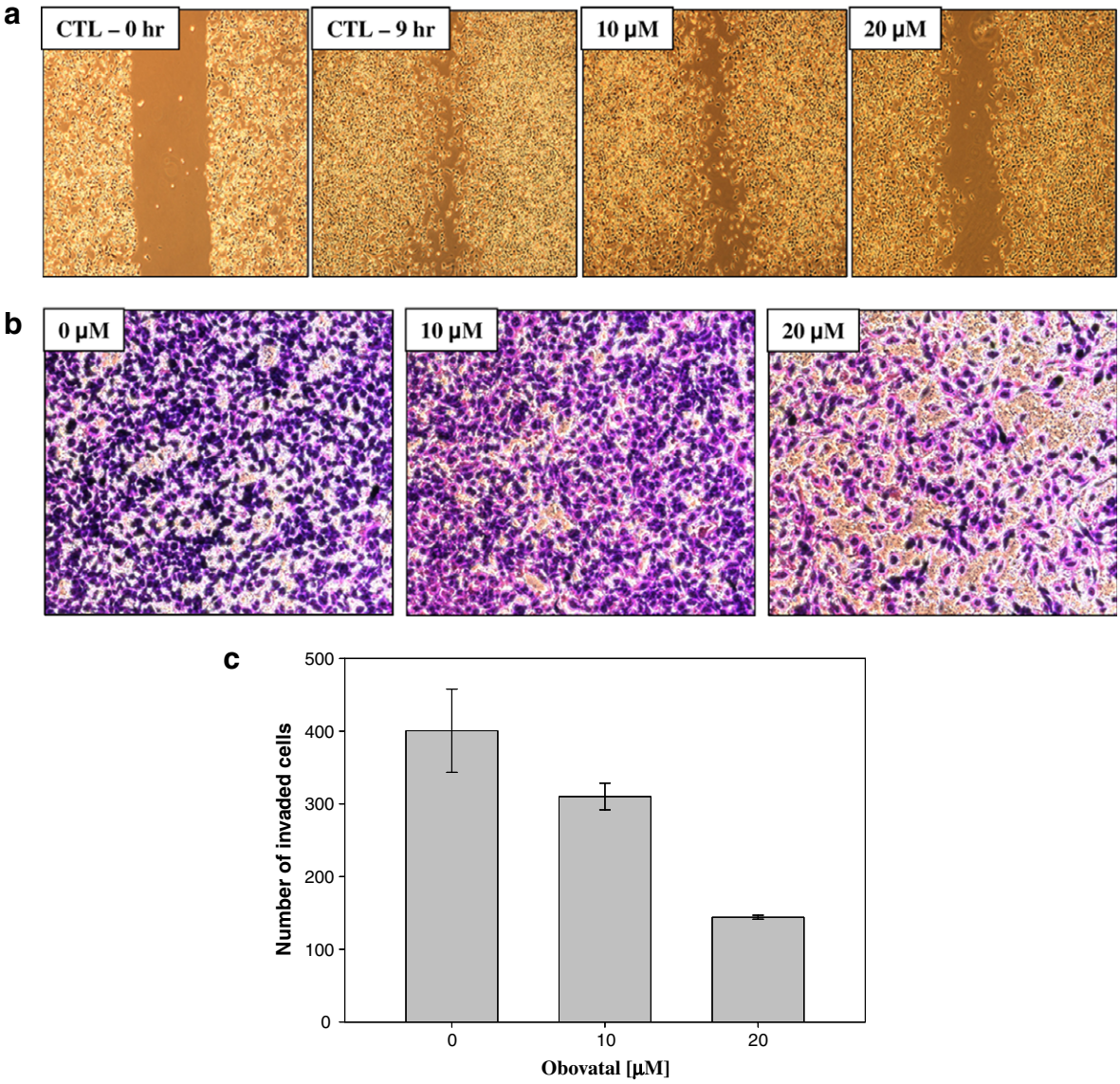

Figure 5. Effect of obovatal on the migration and invasion in HT1080 cells. (a) HT1080 cells were seeded on 60 mm plates and allowed to grow to confluence. Confluent monolayers were scratched with a pipette tip and maintained for 9 h with/without obovatal. (b) 5×10^4 cells per cell line were trypsinized, washed, and resuspended in serum-free medium and placed in the top portion of the invasion chamber. The lower portion of the chamber contained 10% FBS as a chemoattractant. Cells were incubated in the presence and absence of the obovatal for 24 h at 37 °C in a humidified incubator with 5% CO_2. Cells that had invaded through the Matrigel were fixed in 100% methanol, stained with 0.5% crystal violet, photographed, and counted under a light microscope. (c) Invasion of HT1080 cells treated with 20 μM obovatal was blocked by more than 70% compared with that of the vehicle treated cells. Average values from three experiments are shown. The error bars represent the standard deviation.

4.4. MMP-2 enzyme assay in vitro

To measure the activity of MMP-2 enzyme in vitro, purified recombinant MMP-2 expressed by insect cells and fluorescent substrate MOCAc-Pro-Leu-Gly-Leu-A2pr (Dnp)-Arg-NH2 were used. Compound (2 μl) was added to wells of microtiter plates, each of which contained 100 μl of TNC buffer (50 mM Tris-HCl, pH 7.5, +150 mM NaCl + 10 mM $CaCl_2$ + 0.02% NaN_3 + 0.05% Brij-35). Fifty microliters of the enzyme solution (5 ng/ml) was added to this solution and preincubated at 37 °C for 10 min. Then, 50 μl of substrate sol. (10 μM) was added to the mixture to begin the reaction. The fluorescence values were measured at an excitation wavelength of 328 nm and an emission wavelength of 393 nm after incubation at 37 °C for 1 h.[22]

4.5. Cells and culture

HFF (human foreskin fibroblast) and various tumor cell lines, SW620 (human colon carcinoma), HCT116 (human colon carcinoma), and HT1080 (Human fibrosarcoma cells), were purchased from ATCC. HFF cells were maintained as monolayer cultures in DMEM supplemented with 10% heat-inactivated FBS (Gibco/BRL). SW620 and HT1080 cells were maintained in RPMI1640, and HCT116 cells were maintained in

McCoy's 5a supplemented with 10% heat-inactivated FBS (Gibco/BRL), 5% sodium pyruvate, non-essential amino acids, and L-glutamine.

4.6. Cell proliferation assay

Cells were seeded at a density of 5000 cells/well in a 96-well microtiter plate. After 24 h, cells were replenished with fresh complete medium containing various concentrations of obovatal or 0.1% DMSO. Viable cells were measured by cell proliferation reagent WST-1 (Roche) after incubating at 37 °C for 48 h in a 5% CO_2 atmosphere. The amount of WST-1-formazan produced was measured at 450 nm by ELISA Reader (Bio-Rad).

4.7. Scratch wound-healing motility assay

HT1080 cells were seeded on 60 mm plates and allowed to grow to confluence. Confluent monolayers were scratched with a pipette tip and maintained for 9 h. For obovatal treatment, cells were treated with various concentrations immediately after wounding throughout the assay period.

4.8. Invasion assay

To evaluate the anti-invasiveness of obovatal, BioCoat Matrigel chambers (Becton Dickinson Labware, Bedford, MA) were used according to the protocol of the manufacturer. Briefly, 5×10^4 cells per cell line were trypsinized, washed, and resuspended in serum-free medium and placed in the top portion of the invasion chamber. The lower portion of the chamber contained 10% FBS as a chemoattractant. Cells were incubated in the presence and absence of the obovatal for 24 h at 37 °C in a humidified incubator with 5% CO_2. Cells that had invaded through the Matrigel and migrated to the bottom chamber were fixed in 100% methanol, stained with 0.5% crystal violet, photographed, and counted under a light microscope (Nikon).

4.9. Zymogram analysis

HT1080 cells were cultured for 24 h in RPMI1640 (10% FBS) containing obovatal and cultured for an additional 12 h in serum-free medium before collection of the conditioned medium for MMP assays. Proteins in the conditioned media were separated with 10% SDS–PAGE containing gelatin. The unstained bands correspond to the areas of collagen digestion.

4.10. RT-PCR (reverse transcriptase-polymerase chain reaction) assays

The cDNA (complementary DNA) was synthesized from a total of 5 μg of RNA using Superscript II reverse transcriptase (Invitrogen). Two microliters was used in a total of 20 μl reaction volume as a template for PCR amplification. PCR was performed under standard conditions in 20 μl; 10 mM Tris, pH 8.3, 40 mM KCl, 1.5 mM $MgCl_2$, 250 μM dNTP (deoxyribonucleotide triphosphate), 10 pM each primer (sense and antisense), and 1 U *Taq* DNA polymerase (Bioneer, Taejon, Korea). The primer sequences for MMP-2 were designed using the primer software at the web site http://frodo.wi.mit.edu/primer3/primer3_code.html. The forward primer (5′-ATGACAGCTGCACCACTGAG-3′) and the reverse primer (5′-GCCTCGTATACCGCAT CAAT-3′) were used to amplify MMP-2 cDNA. Quantity analysis is based on the intensity of the RCR product using the Quantity ONE software (Bio-Rad).

4.11. Western blot analysis

A 40 μg protein was resolved by 10% SDS–polyacrylamide gel and transferred to the PVDF (polyvinylidene difluoride) membrane (Roche, Germany). The membrane was blocked with TBS-T (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) containing blocking reagents (Roche) for 1 h. MMP-2 and actin primary antibody were purchased from Cell Signaling Technology. The antibodies were used at dilutions recommended by the manufacturers. The membrane was incubated with MMP-2 antibody for overnignt at 4 °C. Incubation in horseradish peroxidase-conjugated secondary antibody (Jackson immunology) was for 1 h at room temperature and then washed three times with TBS-T, and visualized with Chemiluminescence POD (peroxidase) reagents (Roche, Germany). Quantity analysis is based on the intensity of the band using the Quantity ONE software (Bio-Rad).

Acknowledgments

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