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# Autocrine motility factor stimulates the invasiveness of malignant cells as well as up-regulation of matrix metalloproteinase-3 expression via a MAPK pathway

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## Abstract

The autocrine motility factor (AMF) is a multifunctional protein that is involved in tumor progression including enhanced invasiveness via induction of matrix metalloproteinase-3 (MMP3). The increase in MMP3 was found in an AMF-high production tumor cell line, and c-Jun, c-Fos and mitogen-activated protein kinases (MAPKs) were also highly phosphorylated compared with the parent line. AMF stimulation induced the rapid phosphorylation of the cellular MAPK cascade and MMP3 secretion, which was blocked using a specific MAPK inhibitor. Results of this study suggest that AMF stimulation stimulates MMP3 expression via a MAPK signaling pathway.

### Keywords

AMF; MMP3; MAPK; JNK; Metastasis

## 1. Introduction

Autocrine motility factor (AMF)/Neuroleukin (NLK)/maturation factor (MF) is an extracellular phospho hexose isomerase (PHI) that is secreted from malignant or neoplastic cells [1–3]. AMF act in a cytokine-like manner via the autocrine motility factor receptor (AMFR) 78 kDa glyco protein (gp78), which is a seven transmembrane glycoprotein [4]. AMF stimulates tumor angiogenesis [5,6], ascite accumulation [7], apoptotic resistance [8–10], cell proliferation [10,11] and matrix metalloproteinase-3 (MMP3) secretion [12] as well as enhanced cell motility. This phenotypic variation is connected to tumor progression and metastasis. Therefore, understanding the mechanism or the cellular signaling pathway of these individual phenotypes is significant for cancer treatment or diagnosis.

Activation of the G-protein is recognized in gp78 mediated cell motil signaling [13,14], and it is reported that protein kinase A-independent tyrosine kinase and protein kinase C (PKC) are involved in AMF-induced cell locomotion [15,16]. This vascular progression occurrs via



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the PKC and phosphatidylinositol 3-kinase (PI3K)-related pathway [6]. PKA and PI3K are also associated with apoptotic resistance via mitogen- activated protein kinase (MAPK) [8,10]. Furthermore, PI3K is involved in cell proliferation via cyclin D1 expression and cyclin-dependent kinase activity [11]. However, the signaling pathway for MMP3 induction by AMF stimulation is still unclear.

MMP3 is a member of the matrix metalloproteinases gene family, which has specificity for various substrates such as type IV collagen, laminin, fibronectin, and a variety of other basement proteins [17,18]. Therefore, MMP3 is a key enzyme of tumor invasion and metastasis with destruction of basement membranes and proteolysis of extracellular matrix.

The promoter region of MMP3 includes a TATA box, TPA response element (TRE) and an Ets site [19,20]; therefore, MMP3 is induced by various cytokines and growth factors such as epidermal growth factor (EGF) [21,22], interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-alpha) [23]. It has been reported that c-Jun N-terminal kinase (JNK)-mediated phosphorylation of c-Jun is triggered by MMP3 induction [24–27]. AMF stimulation also induces JNK phosphorylation via activation of small GTPase Rho [14]. Therefore, it seems likely that the induction of MMP3 by AMF stimulation may occur via a JNK cascade, which we demonstrate in this article.

## 2. Materials and methods

### 2.1. Chemicals and antibodies

JNK inhibitor II and JNK inhibitor II negative control (Merck, Darmstadt, Germany) were used. All reagents and mediums for cell culture were purchased from Gibco, Invitrogen (Grand Island, NY).

The recombinant human AMF was purified by a Glutathione Sepharose® column with PreScission protease (Pharmacia, Uppsala, Sweden).

Mono-specific polyclonal antiserum anti-rhAMF [5–8] and antigp78 [2] were used. Anti-rhAMF rabbit IgG was purified using a ProteinG Sepharose® column (Pharmacia). Sheep anti-human MMP3 was obtained from AbD Serotec (Oxford, UK). Rabbit anti phospho-cJun (S 63/73) and JNK1 (T 183/Y 185) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit anti phospho-cFos (T 325) (BioSource International Inc., Camarillo, CA), and rabbit anti beta actin (BioLegend, San Diego, CA) were used. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-sheep IgG antibodies were purchased from ZYMED Laboratories, Invitrogen (Carlsbad, CA). The human phospho-MAPK Array Kit was obtained from R&D Systems (Minneapolis, MN).

### 2.2. Cell cultures

Human fibrosarcoma *HT-1080* and human acute monocytic leukemia *THP-1* cell lines were provided by RIKEN BRC Cell Bank (Tsukuba, Japan). AMF expression vector and mock vector transformed *HT-1080* cells (*HT-1080*/AMF and *HT-1080*/vector) [5–8] were used. These *HT-1080* cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) supplemented with non-essential amino acids; cells were harvested and passaged for experiments with 0.25% trypsin and 0.025% EDTA. The *THP-1* cells were cultured in RPMI-1640 medium containing 10% FBS. Cultures were maintained at 37 °C in an air–5% $CO_2$ incubator at constant humidity; viability was monitored by trypan blue exclusion. To ensure maximal reproducibility, cultures were grown for no longer than six passages after recovery from frozen stocks and monitored to prevent mycoplasma contamination.

### 2.3. Semi-quantitative RT-PCR

Total RNA was prepared by RNA Isolation System (Promega, Madison, WI) from cultured cells. The cDNA was synthesized by a reverse transcriptase from total RNA using oligo dT primer of a first-stranded cDNA Synthesis Kit (Takara Co, Osaka, Japan). Then, the target mRNA was amplified by PCR with specific primers "dAATGCAGAGACGGCGAAGGAG" and "dTGACTCCCCCTTTCTCTTCTCGT" for human AMF [5–8], "CGCTTGCTGTGTTTTGATGT" and "TCATTGTTGACAGCCAGCTC" for human gp78 [5–8], "dGGTCACTTCAGAACCTTTCCTG" and "dAGTAACTTCATATGCGGCATCC" for human MMP3, "dTGACGGGGTCACCCACACTGTGCCCATCTA", and "dCTAGAAGCATTTGCGGTGGACGATGGAGGG" for human beta actin [5–8]. These primers were designed by Primer3 ver.0.4.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The PCR conditions were as follows: 94 °C for 5 min for initial denaturation, 30 cycles of 94 °C for 30 s, 55 °C for 60 s, 72 °C for 2 min, and 72 °C for 10 min for final extension. PCR products were then subjected to 1.0% agarose gel electrophoresis.

### 2.4. Western blotting

The various treated cells ($5 \times 10^6$) were washed with PBS twice and lysed by 400 μl of lysis buffer [10 mM HEPES pH 7.8, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.2% Tween 20, protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Mannheim, Germany)]. Protein concentration was measured by Bio-Rad protein Assay Kit (Bio-Rad Lab., Hercules, CA). Fifteen micrograms of lysate sample was boiled with 5 × SDS sample buffer (50 mM Tris pH8.0, 5 mM EDTA, 5% SDS, 48% glycerol, 1% 2-mercaptoethanol). All samples were separated by 10% SDS–PAGE and blotted onto PVDF-membranes (ATTO Co., Tokyo, Japan). The blots were blocked with 5% low-fat dry milk in PBS containing 0.02% Tween 20 for 1 h at room temperature. The blocked membrane was incubated with the anti-rhAMF (1/500), anti-gp78 (1/500), anti MMP3 (1/500), anti phospho-cJun (1/200), anti phospho-cFos (1/1000), anti-phospho-JNK1 (1/200) and anti beta actin (1/500) for 1 h, and then with HRP-conjugated anti-rabbit or anti-sheep (1/2000) secondary antibody for 1 h. The labeled bands were revealed by 3,3,5,5-tetramethylbenzidine (TMB) staining.

The human phospho-MAPK array analysis was carried out according to the manufacturer's instructions. The signals were visualized by TMB staining.

### 2.5. MMP3 enzymatic activity

Secreted MMP3 was detected by casein zymogram. The conditioned medium of AMF-treated cells was concentrated to 200-fold by Microcon® YM-30 centrifugal filter devices (MILLIPORE, Bedford, MA), then applied to 10% polyacrylamide gel containing 0.1% casein. Samples were electrophoresed at 4 °C under a non-reducing condition. The gel was washed with 50 mM Tris pH 7.5, 2% Triton X-100 for 30 min twice, and 50 mM Tris pH 7.5 for 5 min twice. The washed gel was incubated at 37 °C in 50 mM Tris pH 8.0 containing 150 mM NaCl, 10 mM $Ca^{2+}$, 1 μM $Zn^{2+}$ for 16 h. Enzymatic activity was visualized as negative staining with Coomassie Brilliant Blue.

### 2.6. Measurement of cell invasiveness

Cell invasiveness was assayed in a Transwell cell culture chamber (24 wells plate) with polyvinylpyrroridone free polycarbonate (PVP) filters (pore size: 8 μm) coated with 5 μg of Matrigel (Collaborative Research Inc., Bedford, MA) on the upper surface. $1 \times 10^5$ of cell suspensions (100 μl) was added to the upper compartment of the Transwell chamber. DMEM (600 μl) was added to the lower compartment and incubated at 37 °C in a 5% $CO_2$

atmosphere for 2 h. The PVP filters were fixed with methanol and stained with hematoxylin–eosin. The cells on the upper surface of the filter were removed by wiping with a cotton swab. The cells that had invaded the filters were manually counted under a microscope at a magnification of 400.

## 3. Results and discussion

### 3.1. MMP3 expression and kinase phosphorylation were enhanced in the AMF-high expression tumor cell line

Two stable transfectant cell clones were isolated [5–8], *HT-1080*/vector as a mock cell line and *HT-1080*/AMF as a human AMF-high expression line, which had the pBK-CMV expression vector only and pBK-CMV-human AMF, respectively. As shown in Fig. 1, the *HT-1080* parent line and *HT-1080*/vector line were very similar, but the over-expression of AMF was manifested by its increased mRNA level (Fig. 1A) and cellular protein (Fig. 1C) in the *HT-1080*/AMF line. The *HT-1080*/AMF line also showed high expression of mRNA (Fig. 1A), and cellular (Fig. 1C) and extracellular (Fig. 1B) MMP3. The latent and an active form of MMP3 are recognized in the extracellular conditioned medium of *HT-1080*, and pro-MMP3 level of *HT-1080*/AMF was high compared with mock and parent line (Fig. 1B).

Furthermore, phosphorylation of c-Jun and c-Fos of *HT-1080*/AMF was much higher compared with the *HT-1080* parent line and *HT-1080*/vector line (Fig. 1C). c-Fos and c-Jun are components of the transcription factor activating protein-1 (AP-1). It is well known that AP-1 regulates the expression of various proteins containing MMP3 via a phorbol ester tumor promoter (TPA) response element (TRE) in the promoter genes. Jun forms homo- and heterodimers, which bind to the TRE, but Fos is active only as heterodimers with Jun. The Jun/Fos heterodimers showed a much higher affinity for the TRE than the Jun homodimer [28–30]. It is likely that the high pro-MMP3 expression in *HT-1080*/AMF depends on phosphorylation of both c-Jun and c-Fos proteins and they should compose the active AP-1.

There are three major pathways in the MAPK cascade, and the Human Phospho-MAPK Array Kit can detect the phosphorylation status of extracellular signal-regulated kinases (ERK1/2), JNK1/2/3, p38 isoforms, Akt (1/2/3), GSK-3 isoforms, p70 S6 kinase, etc. The ERK1/2 and JNK1/2/3 of *HT-1080*/AMF line was highly phosphorylated compared with *HT-1080*/vector line (Fig. 1D), but there were no differences in phosphorylation status of other intracellular kinases detected by the Human Phospho-MAPK Array Kit (data not shown). ERK1/2 mediates the transcriptional activation of c-Fos by directly phosphorylating the carboxyl terminal domain of c-Fos [31,32], and c-Jun is a major substrate of JNK [33,34]. Therefore, the increased phosphorylation of ERK and JNK molecule in the *HT-1080*/AMF cells does not contradict the activation of c-Fos/c-Jun and up regulation of pro-MMP3.

### 3.2. The AMFR is required for over expression of MMP3 by AMF stimulation

Cell motility stimulation, apoptotic resistance and cell proliferation activity are initiated by AMF binding to its receptor gp78, which is a gp78 present on the cell surface. gp78 was identified as a sialylated peanut agglutinin binding fraction of malignant cell membranes [35,36], and its expression level was found to be increased in a number of different malignancies, correlating with the metastatic potential [37–39]. The binding between AMF and gp78 occurs via a side chain of gp78 [40], and Rho activation signaling connects JNK phosphorylation [14].

We have previously described that some leukemic cell lines do not express gp78 [41]. The human acute monocytic leukemia *THP-1* line also showed reduced gp78 mRNA and protein levels (Fig. 2A). This *THP-1* line did not respond to AMF stimulation, that is the expression

level of MMP3 was not altered by AMF exposure (Fig. 2B, lanes 1–4), whereas obvious over-expression of MMP3 was recognized in the *HT-1080* line exposed to 25 ng/ml AMF (Fig. 2B, lane 8). These data suggest that the pro-MMP3 up-regulation by AMF stimulation also required gp78.

### 3.3. Time-dependent activation of signaling molecule and MMP3 expression

JNK, c-Fos and c-Jun were clearly phosphorylated in the *HT-1080*/AMF line. Therefore, the phosphorylation status of this intracellular signaling molecule was investigated using the *HT-1080* parent line. The cells were stimulated by 25 ng/ml of AMF for 0, 10, 30, 60, 180, and 360 min, then subjected to each experiment.

As shown in Fig. 3, activation of the signaling molecule is recognized at an early stage. Clearly, phosphorylation of JNK1 was observed at 10 min after stimulation, and phosphorylation of c-Fos and c-Jun was also observed within 30 min. The over-expression of MMP3 mRNA occurred at 60 min, and an increase in cellular and extracellular pro-MMP3 was observed at 180 min. This activation of signaling molecules was matched with stimulation by EGF [21,22], IL-1 and TNF-alpha [23].

### 3.4. MMP3 induction by AMF stimulation is depended on JNK pathway

Anthra [1,9-cd] pyrazol-6 (2H)-one; 1,9-pyrazoloanthrone (JNK inhibitor II) is a specific inhibitor for the JNK family. The IC50 is 0.04 μM for JNK1/2, and 0.09 μM for JNK3. N1-Methyl-1,9-pyrazoloanthrone (JNK inhibitor negative control) is a negative control for JNK inhibitor. The IC50 is 18 μM for JNK1/2, and 24 μM for JNK3 [42,43].

The *HT-1080* parent line was exposed to 0, 50, 100, and 200 nM of JNK inhibitor and 200 nM of JNK inhibitor negative control for 6 h. Then, the treated cells were stimulated by 25 ng/ml of AMF for 6 h, and subjected to each analysis.

As shown in Fig. 4, mRNA expression and protein induction of MMP3 by AMF stimulation was clearly exhibited by treatment with JNK inhibitor II (Fig. 4, lanes 3–5), whereas JNK inhibitor negative control did not affect for pro-MMP3 production (Fig. 4, lane 6). JNK inhibitor II also impaired the phosphorylation of c-Jun. These data strongly suggest that the conventional JNK signaling pathway mediates the up-regulation of MMP3 by AMF stimulation. The AMF-induced JNK activation is also connected to the enhancement of cell motility via phosphorylation of myosin light chain kinase [14]. The MAPK pathway has a significant role in regulating the expression of various genes including MMP7 [44], MMP9 [45], MMP13 [46]. Therefore, further studies are needed to investigate the effect of AMF stimulation on regulation of these molecules.

### 3.5. Anti-AMF treatment can regulate MMP3 induction

We have previously established that the anti human AMF polyclonal IgG can exhibit AMF activity, including cell motility [5], angiogenesis [5], hyperpermeability of ascite fluid [7] and apoptotic resistance [8]. Therefore, we investigated the effect of antibody against MMP3 induction.

Cultured *HT-1080*/AMF cells were washed with PBS twice and fresh medium with 0.1, 0.5, and 1 μg/ml or without (as a control) anti AMF IgG was added, and cells were cultured for a further 24 h, then subjected to each experiment. As shown in Fig. 5A, mRNA expression and enzymatic activity of MMP3 was decreased by treatment with a high antibody concentration.

*HT-1080* ($1 \times 10^5$) cells and variant lines were treated with 1 μg/ml anti AMF IgG for 24 h and were applied to the invasion assay. A clear reduction of invasiveness on *HT-1080* parent and *HT-1080*/vector lines with anti AMF IgG treatment was not observed, but significant inhibition was observed with the IgG treated *HT-1080*/AMF line (Fig. 5B). Cell invasion is a complicated phenomenon that includes several steps including adhesion to the basement membrane, enzymatic breakdown of the matrix and migration to the extracellular space. Excess AMF enhances protease activity and cell motility; therefore, neutralization of AMF might be effective for fixing the *HT-1080*/AMF line.

The AMF high expression variant line *HT-1080*/AMF is an example of tumor progression, and it shows various malignant phenotypes including MMP3 upregulation. Therefore, targeting the AMF of malignant cancer by using antibodies or chemicals [1,5,47,48] should effectively support cancer treatment.

## Abbreviations

| | |
|---|---|
| **AMF** | autocrine motility factor |
| **AMFR** | Autocrine motility factor receptor |
| **gp78** | 78 kDa glyco protein |
| **MMP3** | matrix metalloproteinase-3 |
| **MAPK** | mitogen-activated protein kinase |
| **JNK** | c-Jun N-terminal kinase |
| **ERK** | extracellular signal-regulated kinases |
| **PKC** | protein kinase C |
| **PI3K** | phosphatidylinositol 3-kinase |
| **AP-1** | activating protein-1 |
| **TRE** | TPA response element |

## References

1. Watanabe H, Takehama K, Date M, Shinozaki T, Raz A. Tumor cell motility factor is the neuroleukin/phosphohexose isomerase polypeptide. Cancer Res. 1996; 56:2960–2963. [PubMed: 8674049]
2. Niinaka Y, Paku S, Haga A, Watanabe H, Raz A. Expression and secretion of neuroleukin/phosphohexose isomerase/maturation factor as autocrine motility factor by tumor cells. Cancer Res. 1998; 58:2667–2674. [PubMed: 9635595]
3. Haga A, Niinaka Y, Raz A. Phosphohexose isomerase/autocrine motility factor/neuroleukin/maturation factor is a multifunctional phosphoprotein. Biochim Biophys Acta. 2000; 1480:235–244. [PubMed: 11004567]
4. Shimizu K, Tani M, Watanabe H, Nagamachi Y, Niinaka Y, Shiroishi T, Ohwada S, Raz A, Yokota J. The autocrine motility factor gene encodes a novel type of seven transmembrane protein. FEBS Lett. 1999; 456:295–300. [PubMed: 10456327]
5. Funasaka T, Haga A, Raz A, Nagase H. Tumor autocrine motility factor is an angiogenic factor that stimulates endothelial cell motility. Biochem Biophys Res Commun. 2001; 284:1116–1125. [PubMed: 11414699]
6. Funasaka T, Haga A, Raz A, Nagase H. Autocrine motility factor secreted by tumor cells upregulates vascular endothelial growth factor receptor (Flt-1) expression in endothelial cells. Int J Cancer. 2002; 101:217–223. [PubMed: 12209971]

7. Funasaka T, Haga A, Raz A, Nagase H. Tumor autocrine motility factor induces hyperpermeability of endothelial and mesothelial cells leading to accumulation of ascites fluid. Biochem Biophys Res Commun. 2002; 293:192–200. [PubMed: 12054583]
8. Haga A, Funasaka T, Niinaka Y, Raz A, Nagase H. The autocrine motility factor signaling induces tumor apoptotic resistance which regulates apoptosome expression. Int J Cancer. 2003; 105:707–714. [PubMed: 14566819]
9. Romagnolia A, Oliverio S, Evangelisti C, Iannicola C, Ippolito G, Piacentinia M. Neuroleukin inhibition sensitises neuronal cells to caspase-dependent apoptosis. Biochem Biophys Res Commun. 2003; 302:448–453. [PubMed: 12615053]
10. Tsutsumi S, Hogan V, Nabi IR, Raz A. Overexpression of the autocrine motility factor/phosphoglucose isomerase induces transformation and survival of NIH-3T3 fibroblasts. Cancer Res. 2003; 63:242–249. [PubMed: 12517804]
11. Tsutsumi S, Yanagawa T, Shimura T, Fukumori T, Hogan V, Kuwano H, Raz A. Regulation of cell proliferation by autocrine motility factor/phosphoglucose isomerase signaling. J Biol Chem. 2003; 278:32165–32172. [PubMed: 12783864]
12. Yu FL, Liao MH, Lee JW, Shih WL. Induction of hepatoma cells migration by phosphoglucose isomerase/autocrine motility factor through the upregulation of matrix metalloproteinase-3. Biochem Biophys Res Commun. 2004; 314:76–82. [PubMed: 14715248]
13. Watanabe H, Carmi P, Hogan V, Raz T, Silletti S, Nabi IR, Raz A. Purification of human tumor cell autocrine motility factor and molecular cloning of its receptor. J Biol Chem. 1991; 266:13442–13448. [PubMed: 1649192]
14. Tsutsumi S, Gupta SK, Hogan V, Collard JG, Raz A. Activation of small GTPase Rho is required for autocrine motility factor signaling. Cancer Res. 2002; 64:4484–4490. [PubMed: 12154059]
15. Kanbe K, Chigira M, Watanabe H. Effect of protein kinase inhibitors on the cell motility by autocrine motility factor. Biochim Biophys Acta. 1994; 1222:395–399. [PubMed: 8038208]
16. Silletti S, Timar J, Honn KV, Raz A. Autocrine motility factor induces differential 12-lipoxygenase expression and activity in high- and low-metastatic K1735 melanoma cell variants. Cancer Res. 1994; 54:5752–5756. [PubMed: 7954394]
17. Woessner JF Jr. Matrix metalloproteinases and the inhibitors in connective tissue remodeling. FASEB J. 1991; 5:2145–2154. [PubMed: 1850705]
18. Matrisian LM. The matrix-degrading metalloproteinases. Bioassays. 1992; 14:455–463.
19. Butticè G, Duterque-Coquillaud M, Basuyaux JP, Carrère S, Kurkinen M, Stéhelin D. Erg, an Ets-family member, differentially regulates human collagenase1 (MMP1) and stromelysin1 (MMP3) gene expression by physically interacting with the Fos/Jun complex. Oncogene. 1996; 13:2297–2306. [PubMed: 8957070]
20. Biondi ML, Turri O, Leviti S, Seminati R, Cecchini F, Bernini M, Ghilardi G, Guagnellini E. MMP1 and MMP3 polymorphisms in promoter regions and cancer. Clin Chem. 2000; 46:2023–2024. [PubMed: 11106348]
21. Kusukawa J, Harada H, Shima I, Sasaguri Y, Kameyama T, Morimatsu M. The significance of epidermal growth factor receptor and matrix metalloproteinase-3 in squamous cell carcinoma of the oral cavity. Eur J Cancer Oral Oncol. 1996; 32B:217–221.
22. Eum SY, Lee YW, Hennig B, Toborek M. Interplay between epidermal growth factor receptor and janus kinase 3 regulates polychlorinated biphenyl-induced matrix metalloproteinase-3 expression and transendothelial migration of tumor Cells. Mol Cancer Res. 2006; 4:361–370. [PubMed: 16778083]
23. MacNaul KL, Chartrain N, Lark M, Tocci MJ, Hutchinson NI. Discoordinate expression of stromelysin, collagenase, and tissue inhibitor of metalloproteinases- 1 in rheumatoid human synovial fibroblasts. J Biol Chem. 1990; 265:17238–17245. [PubMed: 1698773]
24. Behera AK, Thorpe CM, Kidder JM, Smith W, Hildebrand E, Hu LT. Borrelia burgdorferi-induced expression of matrix metalloproteinases from human chondrocytes requires mitogen-activated protein kinase and janus kinase/signal transducer and activator of transcription signaling pathways. Infect Immun. 2004; 72:2864–2871. [PubMed: 15102798]
25. Park CH, Lee MJ, Ahn J, Kim S, Kim HH, Kim KH, Eun HC, Chung JH. Heat shock-induced matrix metalloproteinase (MMP)-1 and MMP-3 are mediated through ERK and JNK activation

and via an autocrine interleukin-6 loop. J Invest Dermatol. 2004; 123:1012–1019. [PubMed: 15610507]

26. Sweeney SE, Hammaker D, Boyle DL, Firestein GS. Regulation of c-Jun phosphorylation by the IkB kinase-£complex in fibroblast-like synoviocytes. J Immunol. 2005; 174:6424–6430. [PubMed: 15879144]
27. Inoue T, Hammaker D, Boyle DL, Firestein GS. Regulation of JNK by MKK-7 in fibroblast-like synoviocytes. Arthritis Rheum. 2006; 54:2127–2135. [PubMed: 16802349]
28. Sambucetti LC, Curren T. The Fos protein complex is associated with DNA in isolated nuclei and binds to DNA cellulose. Science. 1986; 234:1417–1419. [PubMed: 3491427]
29. Distel RJ, Ro HS, Rosen BS, Groves DL, Spiegelman BM. Nucleoprotein complexes that regulate gene expression in adipocyte differentiation: direct participation of c-Fos. Cell. 1987; 49:835–844. [PubMed: 3555845]
30. Bhomann D, Bos TJ, Admon A, Nishimura T, Vogt PK, Tjian R. Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science. 1987; 238:1386–1392. [PubMed: 2825349]
31. Deng T, Karin M. c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from JNK and ERK. Nature. 1994; 371:171–175. [PubMed: 8072547]
32. Monje P, Marinissen MJ, Gutkind JS. Phosphorylation of the carboxyl-terminal transactivation domain of c-Fos by extracellular signal-regulated kinase mediates the transcriptional activation of AP-1 and cellular transformation induced by platelet-derived growth factor. Mol Cell Biol. 2003; 23:7030–7043. [PubMed: 12972619]
33. Nishida E, Gotoh Y. The MAP kinase cascade is essential for diverse signal transduction pathways. Trends Biochem Sci. 1993; 18:128–131. [PubMed: 8388132]
34. Ip YT, Davis RJ. Signal transduction by the c-Jun N-terminal kinase (JNK)-from unflammation to development. Curr Opin Cell Biol. 1998; 10:205–219. [PubMed: 9561845]
35. Nabi IR, Raz A. Cell shape modulation alters glycosylation of a metastatic melanoma cell-shape antigen. Int J Cancer. 1987; 40:396–402. [PubMed: 3623718]
36. Nabi IR, Raz A. Loss of metastatic responsiveness to cell shape modulation in a newly characterized B16 melanoma adhesive variant. Cancer Res. 1987; 48:1258–1264. [PubMed: 3342405]
37. Watanabe H, Nabi IR, Raz A. The relationship between motility factor receptor internalization and the lung colonization capacity of murine melanoma cells. Cancer Res. 1991; 51:2699–2705. [PubMed: 2021948]
38. Benlimame N, Le PU, Nabi IR. Localization of autocrine motility factor receptor to caveolae and clathrinindependent internalization of its ligand to smooth endoplasmic reticulum. Mol Biol Cell. 1998; 9:1773–1786. [PubMed: 9658170]
39. Wang HJ, Benlimame N, Nabi IR. The AMF-R tubule is a smooth ilimaquinone-sensitive subdomain of the endoplasmic reticulum. J Cell Sci. 1997; 110:3043–3053. [PubMed: 9365274]
40. Haga A, Tanaka N, Funasaka T, Hashimoto K, Watanabe H, Nakamura KT, Raz A, Nagase H. The autocrine motility factor (AMF) and AMF-receptor combination needs sugar chain recognition ability and interaction using the Cterminal region of AMF. J Mol Boil. 2006; 358:741–753.
41. Haga A, Komazaki S, Funasaka T, Hashimoto K, Yokoyama Y, Watanabe H, Raz A, Nagase H. AMF/G6PI induces differentiation of leukemic cells via an unknown receptor that differs from gp78. Leuk Lymphoma. 2006; 47:2234–2243. [PubMed: 17071500]
42. Lee YJ, Shukla SD. Pro- and anti-apoptotic roles of c-Jun N-terminal kinase (JNK) in ethanol and acetaldehyde exposed rat hepatocytes. Eur J Pharmacol. 2005; 31:31–45.
43. Korhonen R, Linker K, Pautz A, Förstermann U, Moilanen E, Kleinert H. Post-transcriptional regulation of human inducible nitric-oxide synthase expression by the Jun N-terminal kinase. Mol Pharmacol. 2007; 71:1427–1434. [PubMed: 17322004]
44. Gaire M, Magbanua Z, McDonnell S, McNeil L, Lovett DH, Matrisian LM. Structure and expression of the human gene for the matrix metalloproteinase matrilysin. J Biol Chem. 1994; 269:2032–2040. [PubMed: 8294454]
45. Sato H, Seiki M. Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. Oncogene. 1993; 8:395–405. [PubMed: 8426746]

46. Uría JA, Jiménez MG, Balbín M, Freije JM, López-Otín C. Differential effects of transforming growth factorbeta on the expression of collagenase-1 and collagenase-3 in human fibroblasts. J Biol Chem. 1998; 273:9769–9777. [PubMed: 9545314]

47. Tanaka N, Haga A, Uemura H, Akiyama H, Funasaka T, Nagase H, Raz A, Nakamura KT. Inhibition mechanism of cytokine activity of human autocrine motility factor examined by crystal structure analyses and site-directed mutagenesis studies. J Mol Biol. 2002; 318:985–997. [PubMed: 12054796]

48. Tanaka N, Haga A, Naba N, Shiraiwa K, Kusakabe Y, Hashimoto K, Funasaka T, Nagase H, Raz A, Nakamura KT. Crystal structures of mouse autocrine motility factor in complex with carbohydrate phosphate inhibitors provide insight into structure activity relationship of the inhibitors. J Mol Biol. 2006; 356:312–324. [PubMed: 16375918]

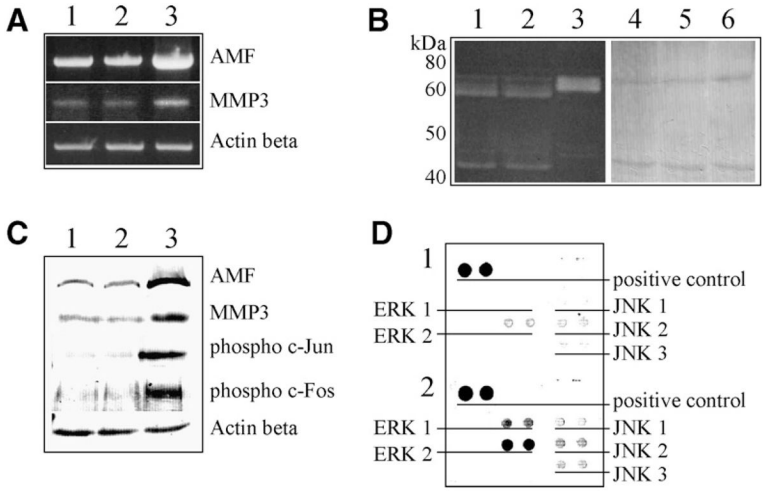


**Fig. 1.**

Comparison of MMP3 expression and MAPK phosphorylation between *HT-1080* and variants. (A) RT-PCR analysis of *HT-1080* and variants. The target cDNA of AMF, MMP3 and β-actin were amplified. Lane 1: *HT-1080*; lane 2: *HT-1080*/vector; lane 3: *HT-1080*/AMF. (B) Extracellular MMP3 of *HT-1080* and variants. Concentrated conditioned medium of each lines were subjected to casein zymogram (lanes 1–3) and Western analysis (lanes 4–6). Lane 1, 4: *HT-1080*; lane 2, 5: *HT-1080*/vector; lane 3, 6: *HT-1080* AMF. (C) Western analysis of *HT-1080* and variants. The AMF, MMP3, phosphorylated c-Jun and c-Fos, and β-actin level of each lysate sample were analyzed by Western method. Lane 1: *HT-1080*; lane 2: *HT-1080*/vector; lane 3: *HT-1080* AMF. (D) The human phospho-MAPK array analysis of *HT-1080*/vector and *HT-1080*/AMF. Panel 1: *HT-1080*/vector; panel 2: *HT-1080*/AMF.

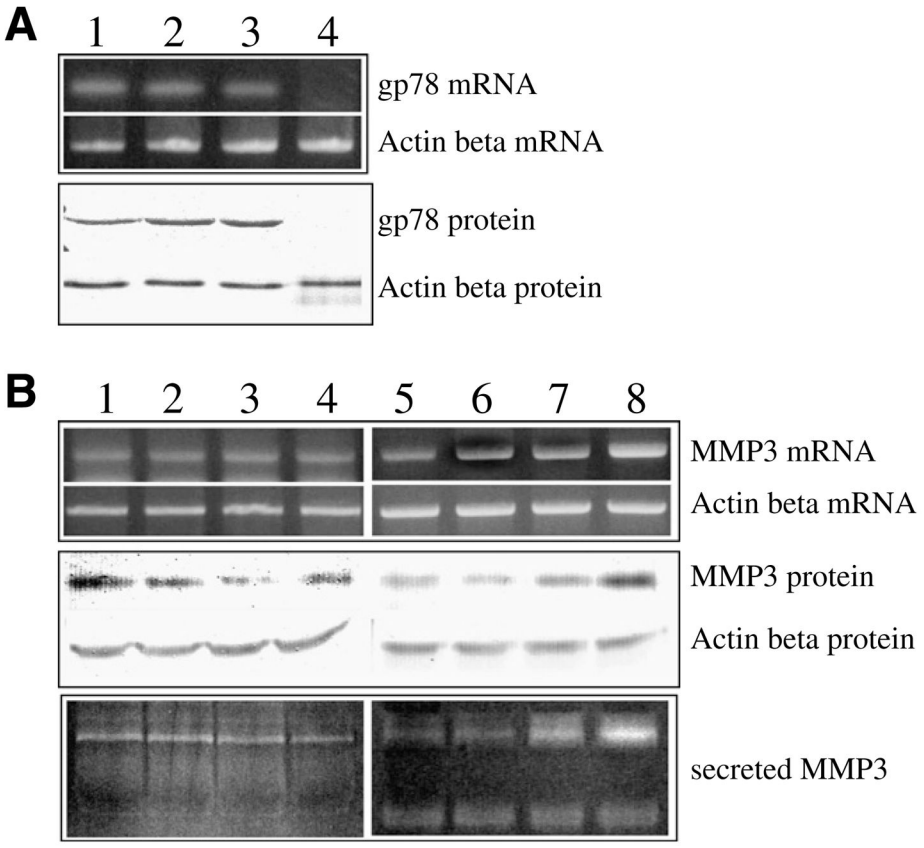


**Fig. 2.**

The gp78 is needed for MMP3 induction by AMF stimulation. (A) The gp78 expression in *HT-1080* and *THP-1* cells. The cDNA of gp78 and β-actin were amplified from each cell lines (upper), and each lysate sample was subjected to Western analysis for gp78 and β-actin (lower). Lane1: *HT-1080*; lane 2: *HT-1080*/vector; lane3: *HT-1080*/AMF; lane 4: THP-1. (B) Both cell lines were exposed to 0, 1, 5, and 25 ng/ml of purified AMF for 24 h. Then, the cells and conditioned medium were subjected to RT-PCR (upper), Western analysis (medium) and casein zymogram (lower) for MMP3 detection. Lanes 1–4; *THP-1*; lanes 5–8: *HT-1080*. Lanes 1, 5: control (0 ng/ml); lanes 2, 6: 1 ng/ml AMF; lanes 3, 7: 5 ng/ml AMF; lane 4, 8: 25 ng/ml AMF.

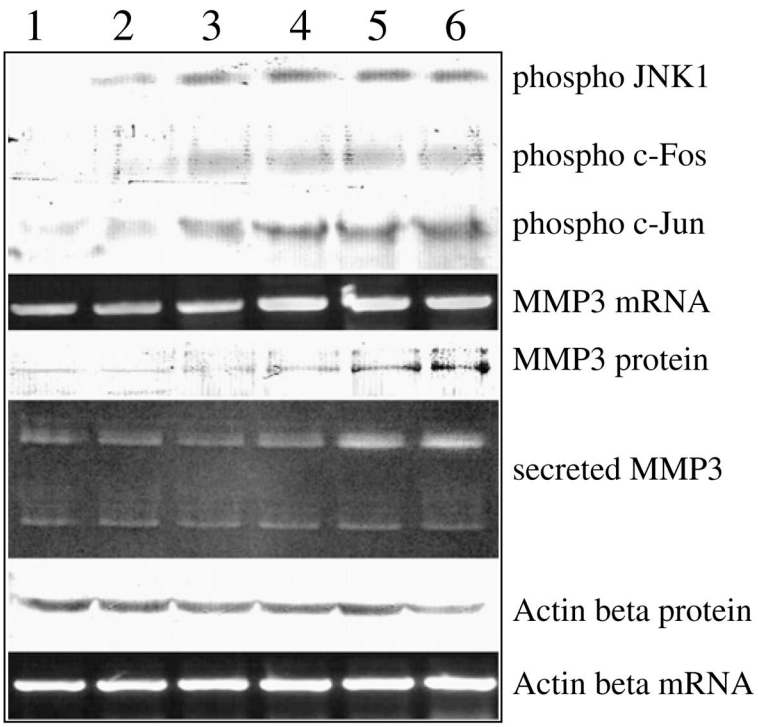


**Fig. 3.**

Time course activation of signaling molecules of *HT-1080* cell line and induction of MMP3 after AMF stimulation. The *HT-1080* cells were stimulated by 25 ng/ml of AMF for 0, 10, 30, 60, 180, and 360 min, then subjected to RT-PCR for MMP3 mRNA, Western analysis for phosphorylation status of signaling molecules and MMP3 expression, and casein zymogram for extracellular MMP3. Lane 1: 0 min; lane 2: 10 min; lane 3: 30 min; lane 4: 60 min; lane 5: 180 min; lane 6: 360 min.

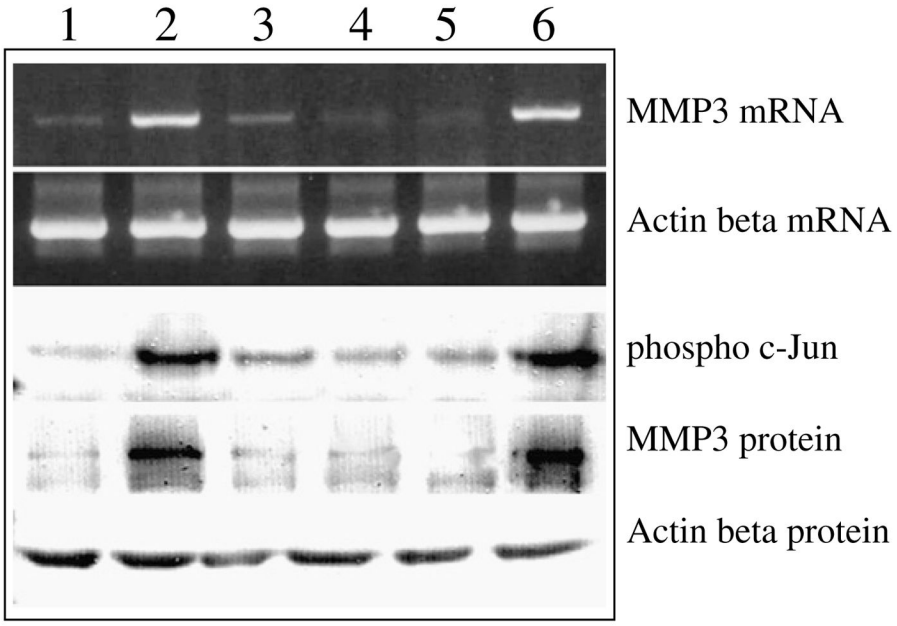


**Fig. 4.**

Pre-treatment by JNK inhibitor exhibits the induction of MMP3 on *HT-1080* cells. The *HT-1080* line was exposed to 0, 50, 100, and 200 nM of JNK inhibitor and 200 nM of JNK inhibitor negative control for 6 h. Then, the treated cells were stimulated by 25 ng/ml of AMF for 6 h, and subjected to RT-PCR for MMP3 mRNA and Western analysis for phosphorylation status of c-Jun and MMP3 expression. Lane 1: control (without AMF addition); lane 2: +25 ng/ml AMF; lane 3: +50 nM inhibitor with AMF; lane 4: +100 nM inhibitor with AMF; lane 5: +200 nM inhibitor with AMF; lane 6: +200 nM inhibitor negative control with AMF.

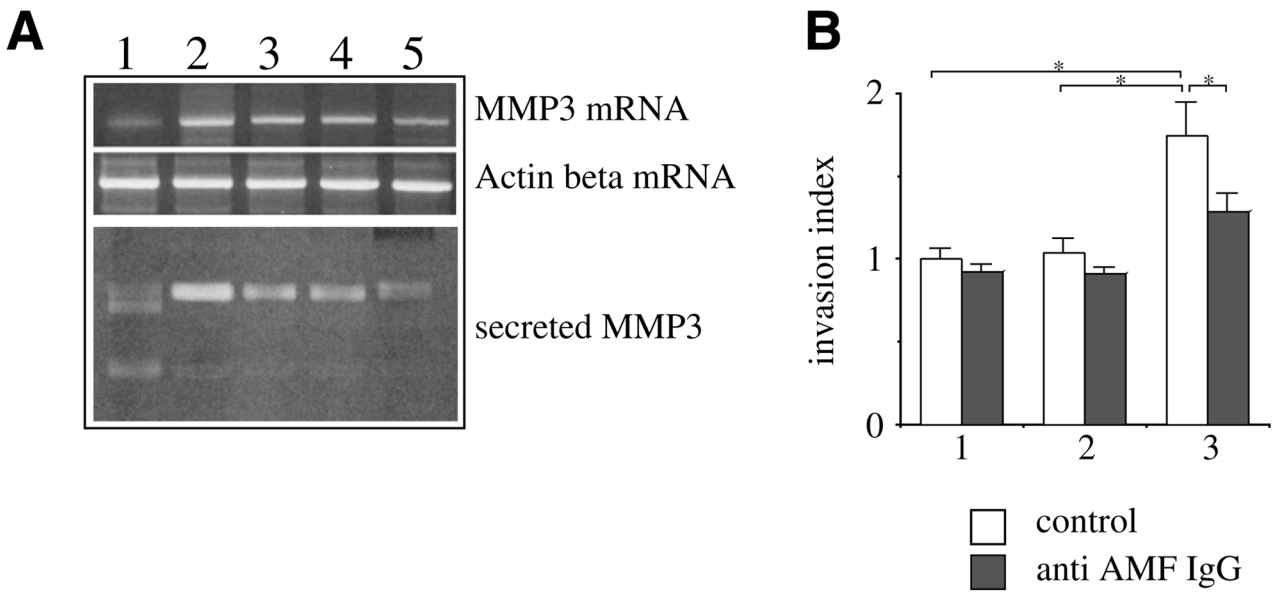


**Fig. 5.**

Anti-AMF IgG exhibits the MMP3 secretion and cell invasiveness. (A) Cultured *HT-1080*/AMF cells were washed with PBS twice and fresh medium containing 0, 100, 500, and 1000 ng/ml anti AMF IgG was added, and cells were cultured for a further 24 h, then subjected to RT-PCR for MMP3 mRNA and casein zymogram for extracellular MMP3. Lane 1: *HT-1080*/vector, without IgG; lane 2: *HT-1080*/AMF, without IgG; lane 3: *HT-1080*/AMF +100 ng/ml IgG; lane 4: *HT-1080*/AMF +500 ng/ml IgG; lane 5: *HT-1080*/AMF +1 μg/ml IgG. (B) Invasiveness of *HT-1080* and variants. *HT-1080* ($1 \times 10^5$) cells and variant lines were treated with 1000 ng/ml anti AMF IgG for 24 hr and were applied to the invasion assay. Column 1: *HT-1080*; column 2: *HT-1080* mock; column 3: *HT-1080* AMF. $^*P < 0.01$ by *t*-test.