Lipid raft localization of epidermal growth factor receptor alters matrix metalloproteinase-1 expression in SiHa cells via the MAPK/ERK signaling pathway

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Received May 24, 2015; Accepted October 3, 2016

DOI: 10.3892/ol.2016.5307

Abstract. Matrix metalloproteinase-1 (MMP-1) has been identified as an important participant in tumor invasion, metastasis and angiogenesis. The purpose of the present study was to investigate the effects of epidermal growth factor receptor (EGFR) localization to lipid rafts on signaling pathways involved in the regulation of MMP-1 expression in SiHa cells, a cervical cancer cell line. EGFR activation by EGF specifically induced MMP-1 expression at both the messenger RNA and protein levels. Additionally, it was observed that EGFR localized to lipid rafts, and that the redistribution of EGFR induced by lipid raft disruption strengthened EGF-induced MMP-1 expression. MMP-1 induction was blocked by the mitogen-activated protein kinase (MAPK) kinase inhibitors PD98059 and U0126. Our results suggested that lipid rafts provide a platform to inhibit EGFR regulation of MMP-1 in SiHa cells through the MAPK/extracellular signal-regulated kinase signaling pathway.

Introduction

Cancer of the cervix occurs in ~500,000 women worldwide each year, with an increased prevalence in relatively young women (1). An essential feature of the progression of cervical cancer is stromal invasion, which is the result of complex, multifactorial processes involving matrix metalloproteinases (MMPs), a closely related multigene family of zinc-dependent proteolytic enzymes (2). Among MMPs, MMP-1 (collagenase-1), together with MMP-8 and MMP-13, are known as the interstitial collagenases, and are capable of initiating the degradation of fibrillar-type collagens by cleaving their N-terminus (3). MMP-1 presents specific substrates for collagenases I, II, III, VII, VIII and X as well as for proteoglycans (4,5). Increased MMP-1 expression has been associated with the incidence or invasiveness of various types of cancer, including colorectal, esophageal, pancreatic, gastric and breast cancer (6-9). Recent studies have demonstrated that the collagenase activity of MMP-1 may be associated with tumor cell invasion and increased angiogenesis in xenograft models of malignant melanomas such as breast cancer (9). Furthermore, MMP-1 has also been shown to liberate signaling molecule precursors, including epidermal growth factor (EGF)-like ligands and transforming growth factor-β, from cell surfaces or the extracellular matrix (ECM) (10,11).

The EGF receptor (EGFR) has been shown to be expressed at moderate-to-high levels in carcinoma of the cervix in addition to a wide variety of other solid tumors (12). Furthermore, an increase in EGFR expression with an increase in disease stage has been observed, and EGFR expression has been associated with poor prognosis (13). EGFR is a receptor tyrosine kinase whose function has been implicated in regulating nuclear and cytoplasmic events, including proliferation, survival, differentiation and migration. Autophosphorylation of EGFR to phosphorylated (phospho)-EGFR leads to the activation of two downstream pathways: The mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3-K)/AKT pathway. The major MAPK pathways consist of extracellular signal-regulated kinase (ERK)1/2, p38 and c-Jun N-terminal kinase (JNK) (14). The sub-cellular localization of EGFR determines the signaling pathways stimulated by EGFR activation (15). The most well-known localization of EGFR is to lipid rafts, which are enriched in cholesterol, sphingolipids and gangliosides, and are less fluid than the surrounding bulk plasma membrane (16). Lipid

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Abbreviations: MMPs, matrix metalloproteinases; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3-K, phosphatidylinositol 3-kinase; JNK, c-Jun N-terminal kinase; MβCD, methyl-β-cyclodextrin; HR-HPVs, high-risk human papillomaviruses

Key words: lipid raft, epidermal growth factor receptor, extracellular signal-regulated kinase, matrix metalloproteinase-1

rafts may act as platforms to facilitate the crosstalk between different components of various signaling pathways that are in close proximity or as sequestering regions to prevent the association of components of signaling events (17,18). However, the effect of EGFR localization to lipid rafts is not well understood. While it has been noted that lipid raft localization of EGFR inhibits ligand binding and subsequent downstream signaling (19,20), other studies have shown that lipid rafts promote EGFR signaling (21).

Our analyses previously demonstrated that EGFR regulates melatonin receptor type 1A (MT1)-MMP and MMP-2 synthesis in the SiHa cervical cancer cell line via both the PI3-K/AKT and MAPK/ERK pathways (22). However, the effects of EGFR localization to lipid rafts on signaling pathways involved in the regulation of MMP-1 expression are unknown. In the present study, it was concluded that lipid raft localization of EGFR alters MMP-1 expression in SiHa cells via the MAPK/ERK signaling pathway.

Materials and methods

Cell culture. SiHa cells were purchased from the Chinese Academy of Sciences cell bank (Shanghai, China). Cells were plated in 6-well plates at ~2x10^6 viable cells per well in 1 ml of Dulbecco's modified Eagle medium (Solarbio Science and Technology Co., Ltd., Beijing, China) containing 10% fetal bovine serum (Zhejiang Tianhang Biotechnology Co., Ltd., Hangzhou, China), and cultured at 37˚C in an atmosphere of 5% CO_2 and 95% air. Cells were allowed to attach for 24 h and then incubated in serum-free medium for 16-18 h. Subsequently, the cells were treated with EGF (PeproTech Inc., Rocky Hill, NJ, USA) in the presence or absence of methyl-β-cyclodextrin (MβCD; 0.5 nM; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), cholesterol (10 μM; Sigma-Aldrich; Merck Millipore) and inhibitors of EGFR (ZD1839; 10 nM; AstraZeneca, London, UK), PI3-K (LY294002; 20 nM; and wortmannin; 5 nM), MAPK kinase (MEK) (PD98059; 20 nM; and U0126; 10 nM), p38 (SB203580; 10 nM) and JNK (SP600125; 10 nM; all Sigma-Aldrich; Merck Millipore) for 1 h prior to exposure to EGF. Cells were harvested after incubation with EGF for the indicated length of time.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Total RNA was reverse-transcribed into single strand complementary DNA using the First Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China). qPCR was performed using the Power SYBR® Green PCR Master Mix on an ABI 7500 Real Time PCR System (both Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec. The sequences of the RT primers were as follows: Human (h) MMP-1 (346 bp) sense 5'-CATCGTGTTGCGGCTCAT-3' and antisense 5'-GCCCAT TTGGCAGTTGTG-3' (59.4˚C); h tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) (285 bp) sense 5'-TCCTGTTGT TGCTGTGGCTGAT-3' and antisense 5'-ACTCCTCGCTGC GGTTGTG-3' (59.4˚C); and hGAPDH (502 bp) sense 5'-GGT GAAGGTCGGTGTGAACGGATTT-3' and antisense 5'-AAT GCCAAAGTTGTCATGGATGACC-3' (58.0˚C). The relative expression level was calculated using the ΔΔCq method (23).

Biochemical lipid raft isolation. Biochemical lipid raft isolation was performed following established protocols (24). Briefly, cells in 6-well plates were scraped in buffer [20 mM Tris (pH 7.8), 250 mM sucrose, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 100 M sodium orthovanadate] and then lysed in buffer containing 1X protease inhibitor cocktail (Solarbio Science and Technology Co., Ltd.) by passing through a 22-gauge needle (Sigma-Aldrich; Merck Millipore) 20 times. Lysates were centrifuged as described (24), and the first and second post-nuclear supernatants were combined and frozen at -20˚C. Samples were thawed and combined with an equal volume of 50% Opti-Prep (Greiner Bio-One, Monroe, NC, USA), and 0-20% Opti-Prep gradient was then applied. Gradients were centrifuged for 90 min at 52,000 x *g* and then fractionated into 12 0.74-ml fractions. Fractions were either dot blotted with cholera toxin subunit B-horseradish peroxidase (1:100 dilution; cat. no. C34780; Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min on ice, and then for 20 min at 37˚C, to determine monosialotetrahexosylganglioside (GM1) expression or subjected to western blotting with antibodies.

Western blotting. Cells were lysed in radioimmunoprecipitation assay lysis buffer plus protease inhibitors (Solarbio Science and Technology Co., Ltd.) and phosphatase inhibitor cocktail (Sigma-Aldrich; Merck Millipore). The concentration of protein in each sample was measured with a BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Aliquots of protein (40 μg) were subjected to western blotting as described previously (25). The following primary antibodies were used: Anti-EGFR (1:1,000 dilution; cat. no. 2239), anti-phospho-EGFR (1:1,000 dilution; cat. no. 2641), anti-AKT (1:2,000 dilution; cat. no. 2920), anti-phospho-AKT (Ser473) (1:1,000 dilution; cat. no. 12694), anti-ERK1/2 (1:1,000 dilution; cat. no. 4348), anti-phospho-ERK1/2 (Thr202/Tyr204) (1:1,000 dilution; cat. no. 14227), anti-p38 (1:1,000 dilution; cat. no. 14451), anti-phospho-p38 (Thr180/Tyr182) (1:1,000 dilution; cat. no. 4092), anti-JNK (1:1,000 dilution; cat. no. 3708), anti-phospho-JNK (Thr183/Tyr185) (1:1,000 dilution; cat. no. 4671; all Cell Signaling Technology, Inc., Danvers, MA, USA), anti-β-actin (1:1,000 dilution; cat. no. SC-130300) and anti-MMP-1 (1:1,000 dilution; cat. no. SC-8836-R; both Santa Cruz Biotechnology Inc., Dallas, TX, USA). The bound antibodies were detected with the appropriate secondary antibodies (1:2,000 dilution; cat. nos. 7074, 7076 and 7077; Cell Signaling Technology, Inc.) and the protein bands were visualized with a 3,3'-diaminobenzidine staining kit (Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China). The bands were quantified using ImageJ 1.37 software (National Institutes of Health, Bethesda, MD, USA).

Immunostaining. Cells were plated on coverslips at a density of 2.0x10^5 cells per 35-mm dish and grown for 48 h in growth medium. Coverslips containing cells were then incubated with 1 mg/ml Alexa Fluor 594-labeled cholera toxin subunit B (red; cat. no. C-34777; Thermo Fisher Scientific, Inc.) for 10 min on ice. Following incubation, cells were fixed with formalin,

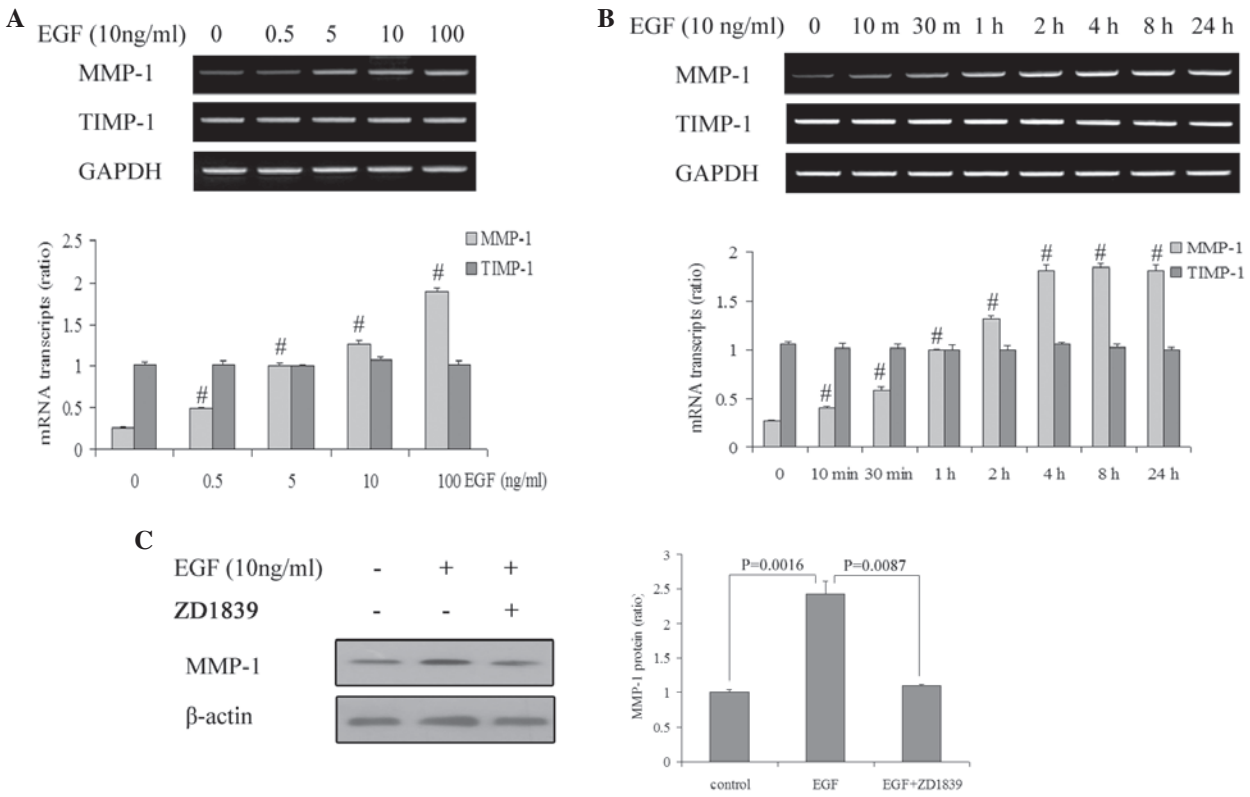

Figure 1. EGF increases MMP-1 expression but does not change TIMP-1 expression. Serum-deprived SiHa cells were incubated with (A) 0.5, 5, 10 or 100 ng/ml EGF for 2 h or (B) with 10 ng/ml EGF for the lengths of time indicated. Total RNA was extracted and analyzed by RT-qPCR. The ΔΔCq results of the RT-qPCR products (data are the mean of three independent experiments ± SD) are shown as the ratio of MMP-1 and TIMP-1 to GAPDH mRNA in the bar graphs. #P<0.05 compared with control (non-stimulated) cells alone. (C) Serum-starved SiHa cells in the presence or absence of the EGFR inhibitor ZD1839 were incubated with 10 ng/ml EGF for 24 h, and the cell lysates were then analyzed by western blotting using anti-MMP-1 or anti-β-actin antibodies as probes. The laser densitometry results (the data are the mean of three independent experiments ± SD) are shown in the bar graph as the ratio of MMP-1 to β-actin (P<0.05; EGF vs. control; EGF+ZD1839 vs. EGF). The results shown are from representative experiments performed in triplicate. MMP, matrix metalloproteinase; EGF, epidermal growth factor; TIMP-1, tissue inhibitor of matrix metalloproteinase-1; SD, standard deviation; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; mRNA, messenger RNA.

permeabilized with 0.1% Triton X-100, blocked in 20% goat serum (Zhejiang Tianhang Biotechnology Co., Ltd.) for 1 h and incubated with anti-EGFR labeled with Alexa Fluor 488 (green; 1:100 dilution; cat. no. A-11008; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at 37˚C. Nuclei were stained with DAPI (blue; Invitrogen; Thermo Fisher Scientific, Inc.). Imaging was performed via confocal microscopy using an Axioplan 2 Apotome microscope (Zeiss GmbH, Jena, Germany) fitted with a 63x1.25 oil immersion lens.

Adenoviral transfections. Cells were plated in 6-well plates at a density of 200,000 cells/ml in triplicates for each condition. The pCMV adenoviruses constitutively active (CA)-MEK and dominant negative (DN)-MEK were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The cells were infected with recombinant adenoviruses to overexpress CA-MEK and DN-MEK at a multiplicity of infection of 25 for 48 h. The medium was then aspirated and replaced with serum-free medium containing MβCD (0.5 mM) for 1 h. Upon incubation, the cells were treated with EGF (10 ng/ml) for 24 h before protein collection.

Statistical analyses. The data were presented as the mean ± standard deviation and subjected to analysis of variance with the Student-Newman-Keuls test using the statistical software package SPSS 11.0 (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

EGF upregulates MMP-1 expression at the messenger RNA (mRNA) and protein levels. It was previously demonstrated by the present authors that EGFR regulates MT1-MMP and MMP-2 synthesis in SiHa cells via both the PI3-K/AKT and MAPK/ERK pathways in SiHa cells (22). To further indicate a role for EGFR in the synthesis and function of other MMP members in SiHa cells, the changes in MMP-1 expression were investigated at the mRNA and protein levels following EGF treatment in the present study. The RT-qPCR results demonstrated that EGF induced an increase in MMP-1 mRNA in SiHa cells in a concentration-dependent manner (P<0.05; Fig. 1A). Additional analysis revealed that the MMP-1 mRNA expression commenced to increase in response to 10 ng/ml EGF (P<0.05), and that it reached maximal levels at 4 h and remained high for ≤24 h (Fig. 1B). However, TIMP-1 mRNA levels remained unchanged by EGF (P>0.05). Furthermore, increased MMP-1 mRNA synthesis was reflected in increased protein levels (P<0.05) that were detectable 24 h after EGF regulation (Fig. 1C).

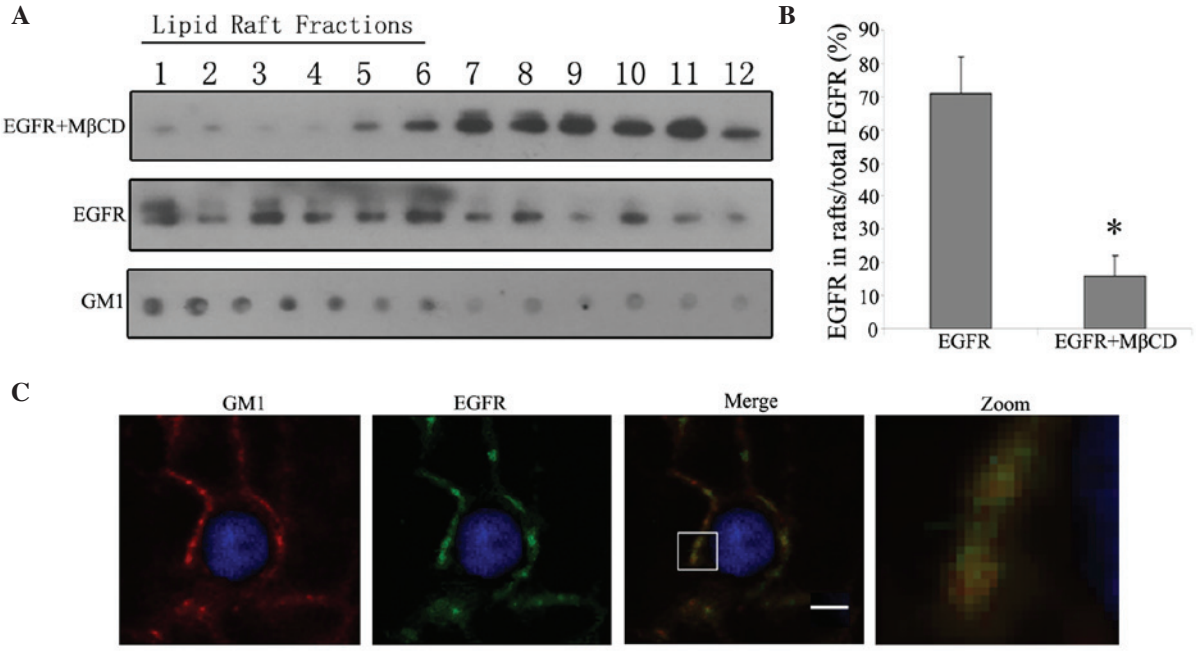

Figure 2. EGFR localizes to lipid rafts in SiHa cells. (A) Cells (1-2x10^6) were plated and cultured for 72 h. Detergent-free lysis was performed, and lipid rafts were separated by ultracentrifugation. Western blotting was performed for EGFR, and fractions were dot blotted for GM1 utilizing cholera toxin subunit B-horseradish peroxidase. Fractions 1-6 contain lipid rafts. Blots are representative of ≥3 independent experiments. (B) Densitometry was performed on western blot images from panel A. Bars represent the percentage of EGFR in lipid raft fractions (1-6) compared with the total amount of EGFR present (fractions 1-12) from ≥3 independent experiments. Statistical analyses were performed utilizing the Student's *t* test (*P<0.05 compared with cells without MβCD treatment). (C) Cells (200,000) were plated onto coverslips and cultured for 48 h. Cells were incubated with Alexa Fluor 594-labeled cholera toxin subunit B at 1 mg/ml for 10 min on ice prior to fixation. Cells were then fixed, blocked in 20% goat serum, and incubated with immunofluorescent anti-EGFR antibodies (green). Nuclei were stained with DAPI (blue). Imaging was performed using a Zeiss Axioplan 2 Apotome microscope fitted with a 63x1.25 oil immersion lens. Magnified views of the boxed area are shown in the bottom panels. Scale bars represent a distance of 5 μm. Images are representative of ≥3 independent experiments. EGFR, epidermal growth factor receptor; MβCD, methyl-β-cyclodextrin; GM1, monosialotetrahexosylganglioside.

To demonstrate that EGFR activation by EGF specifically regulates MMP-1 expression, the activation of EGFR was inhibited using the small molecule inhibitor ZD1839. Addition of ZD1839 (10 nM) 1 h prior to treatment with EGF completely inhibited the EGF-induced increase in MMP-1 at the protein level (P<0.05; Fig. 1C).

EGFR localizes to lipid rafts in SiHa cells. Previous studies have shown that EGFR localizes to lipid rafts in CHO and HeLa cells (24). To determine whether EGFR localizes specifically to lipid rafts in SiHa cells, two methods were used to identify these structures: Biochemical raft isolation and confocal microscopy. First, a detergent-free Opti-Prep gradient was used to isolate lipid rafts (26). Dot blotting for the lipid raft-specific glycosphingolipid GM1 identified fractions 1-6 as lipid raft fractions. When these fractions were immunoblotted using anti-EGFR antibodies, EGFR localization to lipid raft fractions was observed to be most prominent in SiHa cells. When MβCD, a cytotoxic cholesterol-sequestering agent, was used to pharmacologically deplete cholesterol from the cells, the protein levels of EGFR were decreased in the lipid raft fractions (Fig. 2A). Quantitative analysis demonstrated that the lipid raft fractions contained significantly more EGFR compared with the non-lipid raft fractions in SiHa cells (P<0.05; Fig. 2B). Cells were stained with Alexa Fluor 488-labeled anti-EGFR antibodies (green) and Alexa Fluor 594-labeled cholera toxin subunit B (red), which binds specifically to GM1 (27), to detect localization of lipid rafts. Using confocal microscopy, it was observed that EGFR (green) co-localized (yellow/orange) with GM1 (red) at the plasma membrane of SiHa cells (Fig. 2C). Taken together, these data suggested that EGFR localizes within lipid rafts in SiHa cells.

Lipid raft disruption reinforces EGFR-induced upregulation of MMP-1 expression. As previously reported, lipid raft localization of EGFR inhibits ligand binding in certain types of cancers, and lipid rafts promote EGFR signaling in other types of cancer (19-21). Since it was noticed that EGFR localizes to lipid rafts in SiHa cells, the present study examined whether the redistribution of EGFR induced by lipid raft disruption reinforces the EGFR-induced upregulation of MMP-1 expression in SiHa cells. Lipid raft disruption by MβCD enhanced the EGF-induced increase in MMP-1 synthesis at both the mRNA and protein levels (P<0.05), and cholesterol post-treatment reversed this change. To investigate whether EGFR activation by lipid raft disruption specifically regulates MMP-1 expression, the activation of EGFR was inhibited with ZD1839. The results revealed that ZD1839 completely inhibited the MβCD-reinforced MMP-1 synthesis induced by EGF at both the mRNA and protein levels (Fig. 3). These data indicate that lipid raft localization of EGFR inhibits EGFR-induced upregulation of MMP-1 expression.

MAPK/ERK signaling is involved in the regulation of MMP-1 expression. Localization of EGFR to lipid rafts has variable

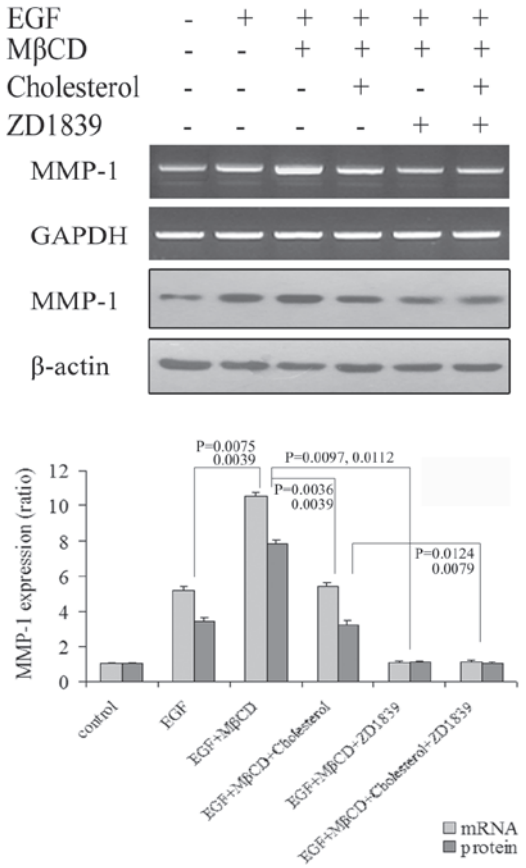

Figure 3. Lipid raft disruption reinforces EGFR-induced upregulation of MMP-1 expression. Serum-deprived SiHa cells were treated with 10 ng/ml EGF in the presence or absence of MβCD, cholesterol and ZD1839 for 1 h before exposure to EGF. Total RNA was extracted 2 h later and then analyzed by reverse transcription-quantitative polymerase chain reaction, in which the MMP-1 or GAPDH mRNA levels were measured. Cell lysates were analyzed 24 h later by western blotting, in which anti-MMP-1 or anti-β-actin antibodies were used as probes. The laser densitometry results (the data are the mean of three independent experiments ± standard deviation) are shown in the bar graph, and are expressed as a ratio. MMP, matrix metalloproteinase; EGF, epidermal growth factor; MβCD, methyl-β-cyclodextrin; mRNA, messenger RNA.

effects on signaling pathways downstream of EGFR (19-21). Thus, the effect of cholesterol depletion on EGFR signaling was examined in SiHa cells. Cells were treated with MβCD, and western blotting was performed to determine whether EGFR induced the phosphorylation of key mediators, including AKT, MAPK, p38 and JNK. As expected, lipid raft disruption resulted in increased AKT, MAPK, p38 and JNK phosphorylation, while cholesterol addition abrogated the MβCD-induced increase in phosphorylation of these kinases (Fig. 4A). To determine the downstream EGFR signaling pathways involved in the increase of MMP-1 expression, SiHa cells were treated with selective PI3-K inhibitors (LY294002 or wortmannin), MEK inhibitors (PD98059 or U0126), a p38 inhibitor (SB203580) or a JNK inhibitor (SP600125) for 2 h before the addition of EGF. The results demonstrated that treatment of SiHa cells with PD98059 or U0126 reduced MMP-1 mRNA expression, which was mediated by both EGF and MβCD ($P<0.05$). By contrast, application of LY294002, wortmannin, SB203580 or SP600125 had no effect on MMP-1 mRNA synthesis ($P>0.05$). Western blot analysis revealed that the impact of these inhibitors on MMP-1 mRNA synthesis induced by both EGF and MβCD was similar to the impact on MMP-1 protein synthesis ($P<0.05$; Fig. 4B). To more closely examine the involvement of the MAPK signaling pathway in the induction of MMP-1 by both EGF and MβCD, adenoviral constructs targeting MEK were employed. The levels of phospho-ERK1/2, total ERK1/2 and MMP-1 were examined following transfection of SiHa cells with a CA-MEK adenoviral construct (Ad-CA-MEK), a DN-MEK adenoviral construct (Ad-DN-MEK) or pCMV control. In the presence of DN-MEK, the phospho-ERK and MMP-1 levels were reduced. In contrast, MMP-1 protein expression was increased in cells transfected with the CA-MEK construct (Fig. 4C). These results indicated that the MAPK/ERK signaling pathway is involved in the regulation of MMP-1 expression by lipid raft disruption.

Discussion

The present study provides evidence describing a role for lipid rafts in the resistance to EGFR-induced MMP-1 expression in SiHa cells. The results demonstrated that EGFR activation by EGF specifically regulates MMP-1 expression at the mRNA and protein levels. Additionally, the current study provided evidence that EGFR localizes to lipid rafts in SiHa cells. In our study, MβCD, a cytotoxic cholesterol-sequestering agent that pharmacologically depletes cholesterol from the cells, decreased EGFR protein levels in lipid raft fractions. Importantly, redistribution of EGFR induced by lipid raft disruption altered MMP-1 expression levels. Furthermore, lipid raft disruption resulted in increased phosphorylation of AKT, MAPK, p38 and JNK. Thus, the MAPK/ERK signaling pathway may be involved in the regulation of MMP-1 expression. Our data suggest that lipid rafts provide a platform to inhibit EGFR regulation of MMP-1 in SiHa cells through the MAPK/ERK signaling pathway.

Invasion and distant metastasis are important events that affect the prognosis and treatment of cervical cancer patients (28). Patients in the later stages of cervical cancer with invasion or metastasis have a significantly worse prognosis (29). Fewer than 20% of women with stage IV cervical cancer survive for ≥5 years (30). Thus, understanding the molecular and cellular mechanisms of cell invasion and metastasis is critical for developing effective cervical cancer therapies and improving patient survival. Cell invasion and metastasis have been associated primarily with degradation of ECM components (26,31). MMP-1 is important in malignant processes of cervical cancer (32). Furthermore, it has become increasingly clear in the past years that MMP-1 substrates extend to numerous non-matrix extracellular and membrane-bound proteins, including protease precursors, protease inhibitors, cytokines, latent growth factors, growth factor-binding proteins and adhesion molecules (9,10,33). Thus, understanding how MMP-1 is regulated in cervical cancer may be crucial for developing more effective therapies for metastatic cancer. The present study examined the influence of EGFR in the regulation of MMP-1 expression. By perturbing EGFR using EGF stimulation in SiHa cervical cancer cells, MMP-1 synthesis was increased. In the same

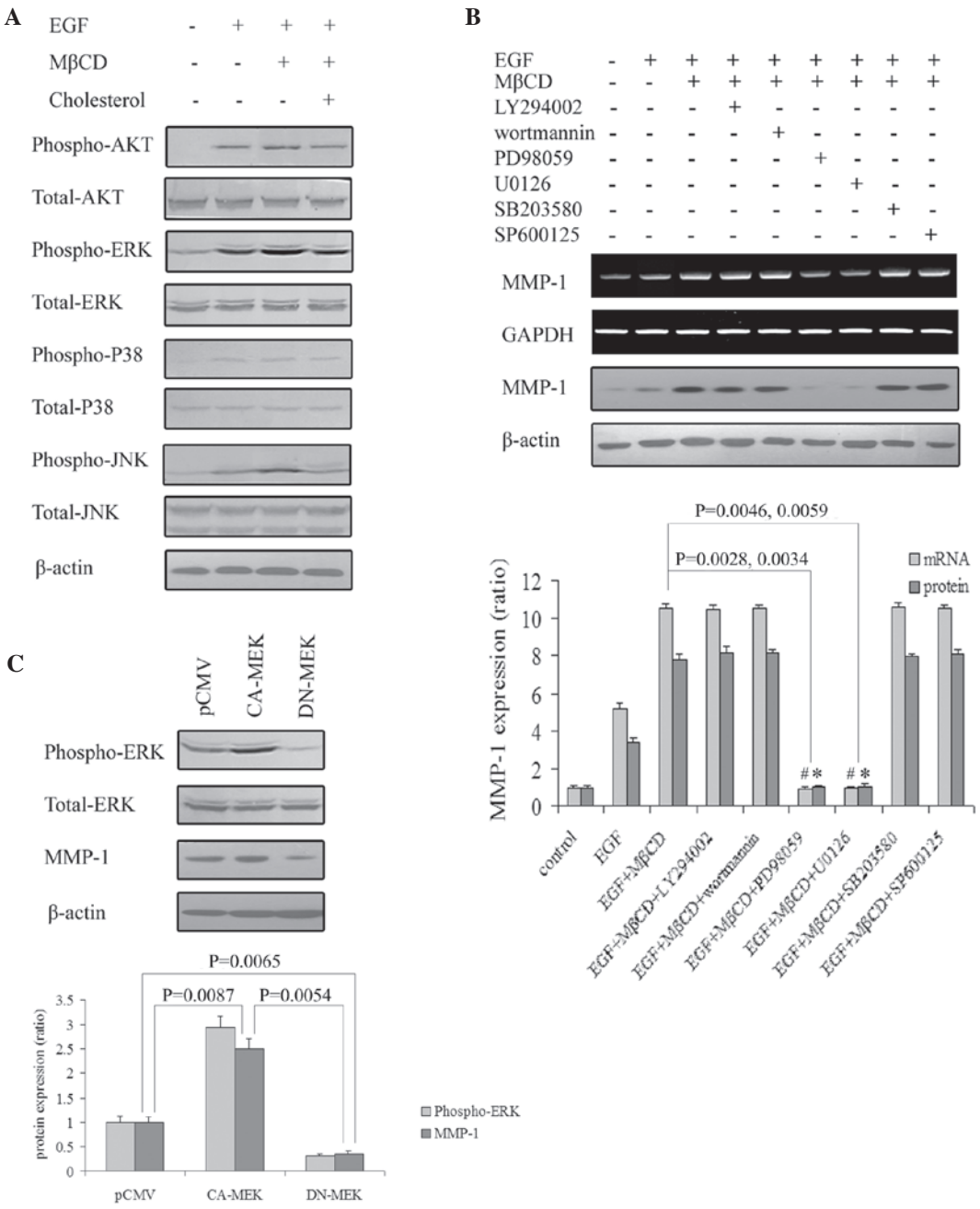

Figure 4. The MAPK/ERK signaling pathway is involved in the regulation of MMP-1 expression. (A) Serum-deprived SiHa cells were incubated for 1 h in the presence or absence of MβCD and cholesterol, and were then incubated with 10 ng/ml EGF for 2 h. The cell lysates were analyzed by western blotting with anti-AKT, anti-phospho-AKT, anti-ERK1/2, anti-phospho-ERK1/2, anti-p38, anti-phospho-p38, anti-JNK, anti-phospho-JNK and anti-β-actin antibodies. The results shown are from representative experiments performed in triplicate. (B) Serum-deprived SiHa cells were incubated with 10 ng/ml EGF in the presence of the corresponding pharmacological inhibitors for 1 h before exposure to EGF. Total RNA was extracted 2 h later and then analyzed by reverse transcription-quantitative polymerase chain reaction, in which the MMP-1 or GAPDH mRNA levels were measured. Cell lysates were analyzed 24 h later by western blotting, in which anti-MMP-1 or anti-β-actin antibodies were used as probes. The laser densitometry results (the data are the mean of three independent experiments ±SD) are shown in the bar graph and are expressed as a ratio. $^{\#}$P<0.05 compared with EGF-stimulated cells with MβCD treatment. *P<0.05 compared with EGF-stimulated cells with MβCD treatment. (C) Cells were infected with recombinant adenoviruses for expression of CA-MEK and DN-MEK for 48 h, and then treated with EGF (10 ng/ml) for 24 h in the absence of MβCD treatment for 1 h before exposure to EGF. Cell lysates were analyzed by western blotting, in which anti-ERK1/2, anti-phospho-ERK1/2, anti-MMP-1 or anti-β-actin antibodies were used as probes. The laser densitometry results (the data are the mean of three independent experiments ± SD) are shown in the bar graphs and are expressed as a ratio. P<0.05 compared with each other. Phospho, phosphorylated; MMP, matrix metalloproteinase; EGF, epidermal growth factor; MβCD, methyl-β-cyclodextrin; mRNA, messenger RNA; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; CA, constitutively active; DN, dominant negative; SD, standard deviation.

model system, inhibition of EGFR by ZD1839 led to decreased MMP-1 levels.

Cervical carcinoma is associated primarily with high-risk human papillomaviruses (HR-HPVs), including HPV-16 and HPV-18, which encode the E6 and E7 oncogenes (34). The E6 and E7 proteins are considered to immortalize cervical epithelial cells by interfering with the function of the tumor suppressor proteins p53 and retinoblastoma protein,

respectively (35). The expression of HR-HPV E6 has been linked to an increase in EGFR levels (13,36), and changes in the functional levels of the HPV E6/E7 proteins may alter the growth rate of cervical cancer cell lines by reducing the stability of EGFR at the post-transcriptional level (37). Thus, it is reasonable to infer that E6/E7 proteins could upregulate the expression of MMP-1 by inducing high levels of EGFR in SiHa cells.

The present data indicate that the localization of EGFR specifically to lipid rafts contributes to the inhibition of the EGFR-induced MMP-1 expression in SiHa cells. EGFR co-localizes with lipid rafts in SiHa cells, and the lipid environment of EGFR-overexpressing cells influences the dimerization properties and signaling functions of EGFR (38). Of note, the 3-hydroxy-3-methyl-glutaryl-coenzyme A-reductase inhibitor statin has been commonly used to deplete cells of lipid rafts for various years (39). Preclinical data have demonstrated that lipid raft depletion by statins can reduce cell growth and sensitize cells to apoptotic stimuli in a number of cancers, including prostate, melanoma and EGFR-overexpressing breast cancer (40,41). Epidemiologic data have demonstrated that the use of statins as single agents in breast cancer is beneficial (42,43). Furthermore, *in vitro* studies combining statins along with other therapies suggest that statins may have a greater clinical benefit when used as part of combinatorial therapies (39). However, the present results indicated that cholesterol depletion synergizes with the activation of EGFR and results in increased phosphorylation of AKT, MAPK, p38 and JNK. It is well known that the activation of two downstream pathways of EGFR, the Ras/Raf/MAPK/ERK pathway and the PI3-K/AKT pathway, can induce cell proliferation and decrease cell apoptosis (44). Importantly, the mechanism of action of statin drugs is not solely through the reduction of cholesterol but also via the inhibition of geranylgeranylated and farnesylated small GTPases, which suppress the activation of small G proteins (45). Therefore, it is difficult to infer whether statins would be beneficial as a part of cervical cancer therapies.

The associations between different signaling pathways and MMPs have been investigated in a number of cell culture systems (46-48). Using selective pharmaceutical inhibitors of EGFR downstream signaling pathway effectors, the present study revealed that MMP-1 expression was upregulated through the MAPK/ERK signaling pathway in SiHa cells treated with EGF and MβCD, and that the PI3-K/AKT, p38 MAPK and JNK/MAPK signaling pathways were not involved in this process. These observations were further supported by the fact that the transfection of MEK-CA led to increased ERK phosphorylation and MMP-1 levels, as well as the fact that the transfection of MEK-DN abolished the basal expression of MMP-1. However, our previous studies indicated that EGFR upregulates MT1-MMP and downregulates MMP-2 through the MAPK/ERK pathway, while concomitantly transmitting a mild positive regulatory signal to the expression of MMP-2 via the PI3-K/AKT pathway in SiHa cells (22). Thus, the signaling pathways involved in the regulation of different MMPs by EGFR are varied.

In conclusion, the present study demonstrated that lipid raft localization of EGFR repressed EGFR-induced MMP-1 expression in SiHa cells, and that the MAPK/ERK signaling pathway was involved in this process. Since MMP-mediated ECM remodeling and invasion of tumors are tightly linked, the regulation of MMP-1 may contribute to the central role that EGFR and lipid rafts have in the development and metastasis of cervical cancer. However, the data presented herein were based on *in vitro* experiments; thus, additional *in vivo* studies are required to obtain a better understanding of MMP-1 involvement in cervical tumorigenesis. Experiments focusing on manipulating MMP-1 expression using an *in vivo* model are in progress and will provide further information regarding the influence of MMP-1 on cervical carcinomas.

Acknowledgements

The present study was supported by the Natural Science Foundation of Heilongjiang Province (Harbin, China; grant no. 200906), the Foundation of Heilongjiang Provincial Educational Department (Harbin, China; grant no. 11551163) and the Foundation of Heilongjiang Provincial Health Department (Harbin, China; grant no. 2009-112).

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