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Pro-MMP-9 upregulation in HT1080 cells expressing CD9 is regulated by epidermal growth factor receptor

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ABSTRACT

Degradation of the surrounding extracellular matrix (ECM) by matrix metalloproteinases (MMPs) drives invasion and metastasis of cancer cells. We previously demonstrated that tetraspanin CD9 expression upregulates pro-MMP-9 expression and release and promotes cellular invasion in a human fibrosarcoma cell line (HT1080). These events were dependent upon the highly functional second extracellular loop of CD9. We report here that the epidermal growth factor receptor (EGFR) tyrosine kinase expression and activity are involved in the CD9-mediated increase in pro-MMP-9 release and cellular invasion. Pro-MMP-9 expression was significantly decreased in a dose-dependent manner using first a broad spectrum receptor tyrosine kinase inhibitor and multiple specific EGFR inhibitors in CD9-HT1080 cells. Furthermore, gefitinib treatment of CD9-HT1080 cells reduced invasion through matrigel. EGFR knockdown using short interfering RNA resulted in decreased pro-MMP-9 expression and release into the media and subsequent cellular invasion without affecting CD9 expression or localization. Conclusively, this study points to EGFR as a key mediator between CD9-mediated pro-MMP-9 release and cellular invasion of HT1080 cells.

1. Introduction

Cancer cell invasion is initiated by matrix metalloproteinase (MMP) degradation of the surrounding extracellular matrix. This process has been extensively researched and subsequently broad spectrum inhibitors were developed to regulate MMP activity *in vivo*. However, these inhibitors demonstrated lack of clinical efficacy and studies were discontinued primarily because pharmacological intervention after metastasis had occurred was ineffective [1–3]. Interest in MMP research has since shifted to finding other ways to regulate their expression, release, and activity prior to metastasis. We recently established that expression of tetraspanin CD9 in a highly invasive human fibrosarcoma cell line (HT1080) specifically augmented the expression and release of a prominent member of the MMP family, pro-MMP-9 [4]. This current study aimed to clarify how CD9 may regulate MMP-9 expression.

Tetraspanins have been implicated in numerous cellular functions including proliferation, migration, and invasion [5]. We previously demonstrated that the highly functional second extracellular loop (EC2) of CD9 was necessary for pro-MMP-9 secretion and subsequent cellular invasion [4]. CD9 EC2 is responsible for a myriad of functions including association with heparin-binding epidermal growth factor-like growth factor [6,7], cell motility [8,9], cell adhesion and fibronectin matrix assembly [10], association with other tetraspanins and integrins [11], and association with TGFα [12]. Interestingly, CD9 has also been demonstrated to associate with epidermal growth factor (EGF) receptor (EGFR) and regulate its activity; however, the mechanisms associated with CD9 regulation of EGFR are not entirely clear. Murayama et al. [13] demonstrated that the EC2 of CD9 associates with EGFR and β1 integrin. Treatment with an anti-CD9 monoclonal antibody, ALB6, enhanced EGFR internalization after EGF treatment [13]. ALB6 has been reported as an inhibitory antibody to CD9 [14,15]; therefore, inhibition of CD9 resulted in increased EGFR internalization. In the same study, CD9 and EGFR expression in Chinese hamster ovary cells (CHO) resulted in increased receptor internalization upon EGF treatment [13]. Hence, the role of CD9 in EGFR regulation is uncertain due to that fact that both inhibition and overexpression had similar effects. Moreover, there is evidence for a lack of association between CD9 and EGFR when CD9 is

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expressed in HEK293 cells [12]. In this latter study, CD9 regulated EGFR activation via its association with transmembrane TGFα in a juxtacrine manner while negatively affecting autocrine TGFα signaling [12].

In a HT1080 model system, CD9 was earlier demonstrated to co-immunoprecipitate with EGFR [16]. Multiple reports suggest that EGFR is strongly connected to the augmentation of MMP-9 expression [17–20]. Because in our studies the absence of CD9 EC2 resulted in an absence of MMP-9 expression, release, and subsequent invasion [4], we hypothesized that EGFR may regulate MMP-9 expression in HT1080 cells. We used multiple EGFR tyrosine kinase inhibitors (TKIs) and siRNA directed to EGFR to explore this phenomenon. We conclude that CD9-mediated pro-MMP-9 expression and release and subsequent cell invasion are dependent upon EGFR expression and activity in HT1080 cells.

2. Materials and methods

2.1. Reagents and antibodies

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) penicillin–streptomycin, Geneticin (G418), human plasma fibronectin (FN), and phosphate buffered saline (PBS; pH 7.4) were purchased from Gibco (Grand Island, NY). Antibodies to anti-human CD9 (mAb7) were generated in our laboratory as previously described [21]. Anti-IgG (M9269), anti-human β-tubulin (T2200), and FITC-conjugated anti-mouse (F2012) antibodies were purchased from Sigma Aldrich (St. Louis, MO). The anti-EGFR antibody (4267) was purchased from Cell Signaling Technologies (Danvers, MA). The horseradish peroxidase (HRP)-conjugated secondary antibodies anti-rabbit (NA934VS) and anti-mouse (NA931VS) were purchased from GE Healthcare (Pittsburgh, PA). Receptor tyrosine kinase inhibitors sunitinib (S-8803), gefitinib (G-4408), erlotinib (E-4997), lapatinib (L-4804) and AG1478 (T-7310) were purchased from LC laboratories (Woburn, MA). Matrigel from Engelbreth-Holm-Swarm mouse tumor and 8.0 μm pore cell culture inserts were purchased from BD Biosciences (Bedford, MA). Lipofectamine 2000 transfection reagent was purchased from Invitrogen (Carlsbad, CA).

2.2. Cell culture

Mock and CD9 HT1080 cells were generated as previously described and cultured in DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, and 0.75 mg/ml G418 in a humidified, 5% CO_2, 37 °C incubator [4]. For each experiment, FN was diluted to 10 μg/ml in PBS and 2 ml was added to each well of 6-well cell culture dishes and allowed to incubate at 37 °C for 3 h. The FN-PBS solution was then aspirated and any residual solution was washed with PBS. Subconfluent HT1080 cells were harvested, counted, and suspended as 5.0×10^5 cells/ml in serum-free (SF) DMEM. 1.0×10^6 cells were added to each FN-coated well in a final volume of 2 ml of SF DMEM and allowed to incubate for 24 h before the media was collected and the cells were harvested.

2.3. Western blot analysis

Cell lysates were harvested using TX-100 lysis buffer (1% TX-100, 20 mM Tris–HCl, 150 mM NaCl, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate) including protease and phosphatase inhibitor cocktails purchased from Sigma Aldrich. Lysate concentration was determined using a colorimetric Bradford Assay and a standard curve. Equal concentrations of lysate mixed with reducing or non-reducing buffer were loaded onto a 12.5% SDS–polyacrylamide gel. The proteins were transferred to a polyvinyl difluoride membrane and non-specific sites of binding were blocked using 5% BSA in Tris-buffered saline with 1% Tween-20 (TBST) solution. Anti-human CD9 (mAb7, 1:500), and-human total EGFR (1:5000), or anti-human β-tubulin (1:20,000) were diluted in 5% BSA-TBST and incubated overnight at 4 °C. After washing off the primary antibody with TBST, a HRP-conjugated anti-mouse antibody (1:5000) for mAb7 or anti-rabbit antibody (1:50,000) for EGFR and β-tubulin were diluted in 5% BSA-TBST, added to the blots, and incubated for 1 h at room temperature. The blots were again washed as described earlier and incubated with Pierce ECL 2 Western blotting substrate (80196, Thermo Scientific, Rockford, IL) for 5 min. X-ray film was used to detect chemiluminesence, and the films were developed using a Konica Minolta X-ray machine. Band density was determined using NIH Image J software, and mAb7 band densities were normalized the corresponding to β-tubulin band densities.

2.4. Receptor tyrosine kinase inhibitor treatment

Each inhibitor was reconstituted in DMSO to the following stock concentrations: sunitinib 50 μM, gefitinib 100 μM, erlotinib 100 μM, lapatininb 100 μM, and AG1478 100 μM. These stock concentrations were further diluted using DMSO and added to the 2 ml of SF DMEM and the cell mixture to get a final concentration of 10, 5, or 1 μM inhibitor with an equivalent final volume of DMSO for each treatment. Likewise, the same volume of DMSO without inhibitor was added to the cells as a negative control. The cells and DMSO or diluted inhibitor were incubated for 30 min at 37 °C, then added to the FN-coated wells and placed in the incubator for 24 h. After 24 h, no difference in cell confluence was observed between the different treatments.

2.5. Gelatin zymography

Gelatin zymography was performed exactly as previously described [4]. Relative density was calculated by dividing by the pro-MMP-9 band in the Mock treatment lane. A minimum of three independent experiments were performed for quantification of relative band density.

2.6. RNA extraction and qRT-PCR analysis

Forward and reverse primers for CD9 and MMP-9 were previously described [4]. EGFR forward: CATGTCGATGGACTTCCAGA and reverse: GGGACAGCTTGGATCA CACT primers were designed using Universal Probe Library primer design tool and purchased from Sigma Aldrich. Total RNA from Mock- and CD9-HT1080 cells was harvested and analyzed exactly as previously described [4]. Sample tests were run in triplicate, and the resulting average cycle threshold (CT) values were normalized to cyclophilin-D housekeeping gene, and fold changes in mRNA expression were calculated using the ΔΔCt method as described [4].

2.7. Flow cytometry

Mock- and CD9-HT1080 cells were harvested and suspended at 5.0×10^5 cells/ml in 5% goat serum-DMEM (blocking media) and incubated on ice for 45 min. All subsequent antibody incubations were performed on ice. Primary antibody was added (5 μg/ml) and incubated for 1 h. Unbound primary antibody was removed by washing the cells three times. Briefly, the cells were pelleted ($800 \times g$ for 5 min), the supernatant was discarded, and the cell pellet was suspended using 1 ml of ice-cold PBS (pH 7.4). The cells were incubated with secondary FITC-conjugated antibody (5 μg/ml) in blocking media for 1 h. The secondary antibody was removed by washing, and the cells were suspended in PBS for data

acquisition. Analysis of the data was performed using a FACS Calibur flow cytometer equipped with Cell Quest Pro software (Becton–Dickinson, Bedford, MA). The geometric mean fluorescence intensity values were averaged among three or more independent experiments.

2.8. Matrigel invasion assay

Matrigel invasion assays were performed exactly as previously described [4]. Cells were counted in 10 random fields of view per membrane and averaged.

2.9. Knockdown of EGFR

Small interfering RNA (siRNA) targeting EGFR was ordered from Cell Signaling Technology (#6480). A non-specific, scrambled siRNA (AM4635) purchased from Ambion (Life Technologies, Grand Island, NY) was used as a control. Prior to transfection, 10 µl of Lipofectamine 2000 was incubated in 0.5 ml of serum-free SmBM for 5 min at room temperature, then 600 pmol of siRNA in 0.5 ml of serum-free SmBM was added to Lipofectamine dilution and allowed to incubate for 20 min. HT1080 cells were 70% confluent upon transfection, cell monolayers were washed three times with PBS, then 1 ml of Lipofectamine-siRNA mixture was added drop-wise to cells in 6-well cell culture plates. After 6 h, the mixture was aspirated and monolayers were rinsed with serum-supplemented SmBM and allowed to incubate for 24–48 h before preforming experiments.

2.10. Statistical analysis

Experiments were repeated at least three independent times. Statistical analysis was carried out using SPSS software, and graphs were generated using GraphPad Prism 6 software. The bars represent the mean, and error bars represent the standard deviation from the mean. An independent samples *t*-test or Mann–Whitney *U* Test was used to compare two means. Analysis of Variance (ANOVA) was used to compare three or more means of normally distributed homogenous data. Tukey's HSD post hoc analysis was conducted to determine any difference among the groups. Welch's ANOVA was used for three sample means with non-normally distributed data, and Dunnet's T3 post hoc analysis was used to determine any difference among these means. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Inhibition of EGFR signaling results in decreased pro-MMP-9 release from CD9-HT1080 cells

Our laboratory previously reported the successful cell surface expression of tetraspanin CD9 in human fibrosarcoma cells that endogenously express low levels of CD9 [4]. The expression of CD9 in CD9-HT1080 cells was confirmed using an anti-human CD9 monoclonal antibody in Western blot (Fig. 1A). The presence of CD9 in the Mock-HT1080 cells was only evident upon prolonged blot exposure (data not shown). CD9-mediated increased expression and release of pro-MMP-9 were also previously demonstrated [4]. This observed increase in pro-MMP-9 release via gelatin zymography was significantly attenuated upon treatment of the cells with a broad spectrum tyrosine kinase inhibitor, sunitinib, and was dose-dependent (Fig. 1B). Quantification of gelatin degradation revealed a significant decrease in CD9-HT1080 cells treated with sunitinib at 5 and 10 µM concentrations.

We further explored the role of EGFR in pro-MMP-9 release using multiple tyrosine kinase inhibitors with previously

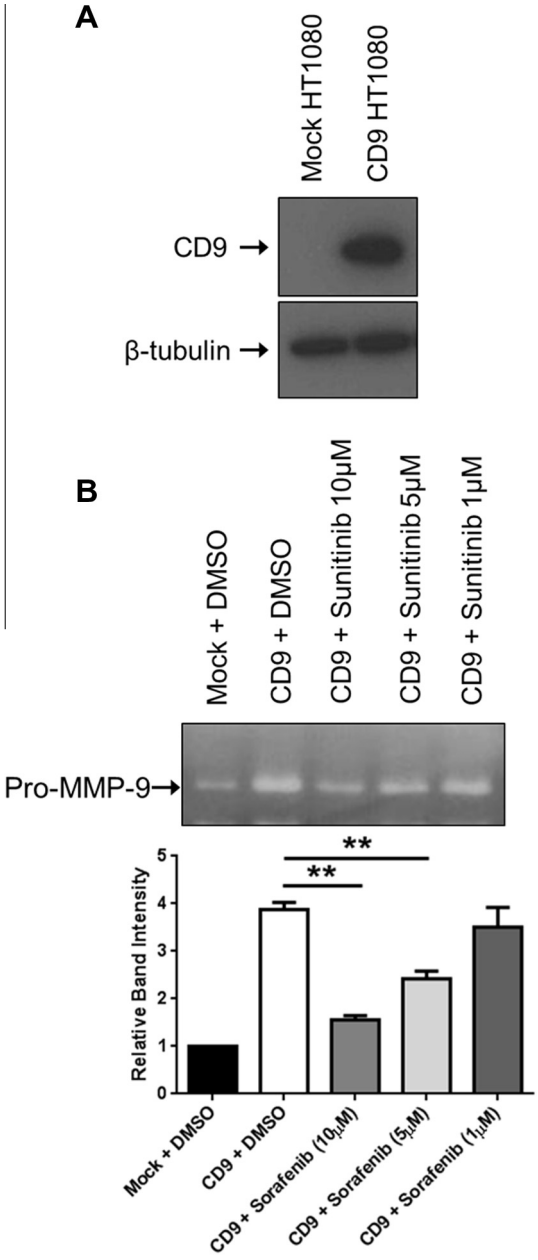

Fig. 1. CD9-HT1080 cells release less pro-MMP-9 after treatment with a broad spectrum receptor tyrosine kinase inhibitor sunitinib. (A) The expression of CD9 in HT1080 cells was assessed by Western blot analysis. (B) Pro-MMP-9 release into serum-free media of HT1080 cells plated on 10 µg/ml FN for 24 h was measured using gelatin zymography as described in Section 2. Gelatinolytic activity was quantitated by measuring band intensity over three independent repeats. Data are presented as mean ± SD ($^{**}p < 0.01$).

demonstrated specificity for EGFR. Getfitnib, erlotinib, lapatinib, and AG1478 all demonstrated a dose-dependent inhibition of pro-MMP-9 release in CD9-HT1080 cells (Fig. 2A–D). Moreover, CD9-HT1080 cells were significantly less invasive through matrigel after treating with 10 µM of gefitinib (Fig. 2E). These findings show that CD9 augments pro-MMP-9 release and subsequent cell invasion through matrigel via EGFR activation.

3.2. Knockdown of EGFR in CD9-HT1080 cells attenuates the release of pro-MMP-9 and subsequent cell invasion

EGFR expression is strongly correlated to increased expression and release of MMP-9 [22,23]. Upon discovering that inhibiting

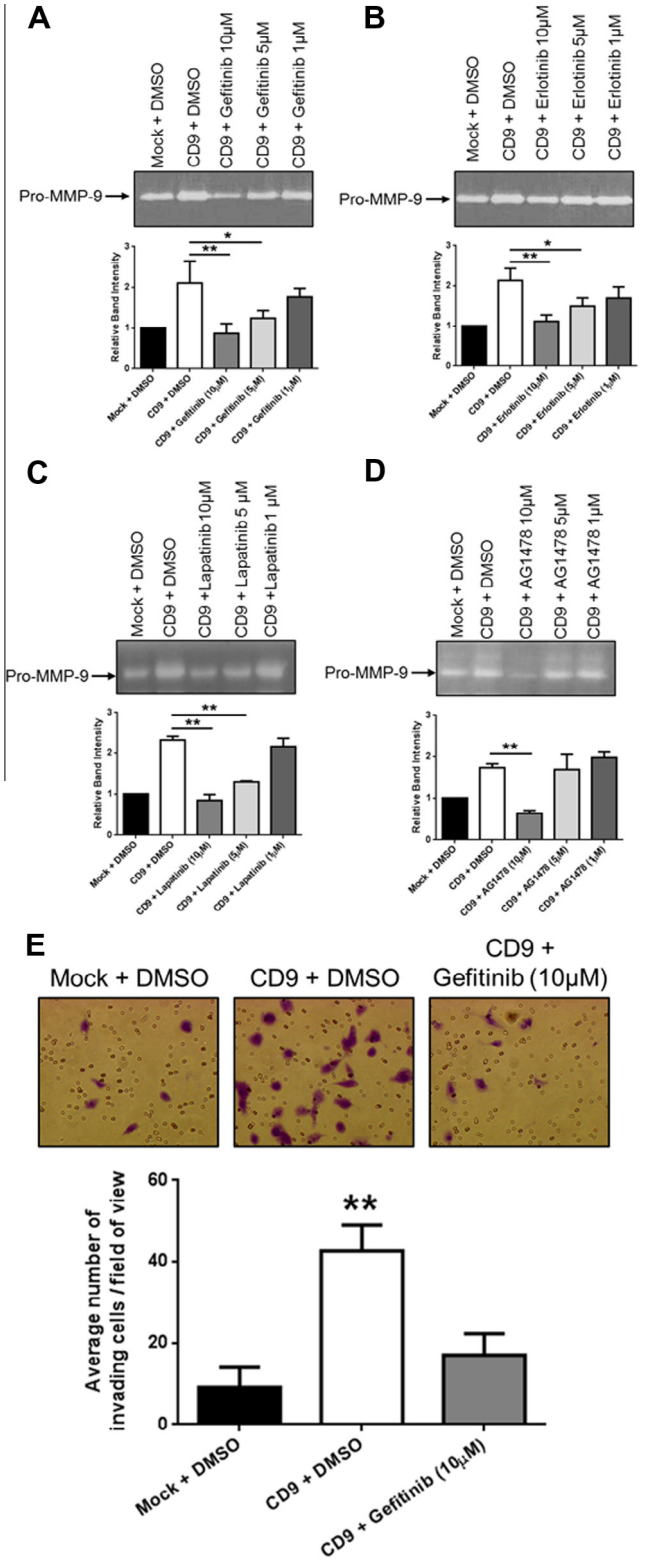

Fig. 2. Epidermal growth factor receptor tyrosine kinase inhibitors reduce the expression of pro-MMP-9 and inhibit CD9-HT1080 cell invasion. (A–D) Pro-MMP-9 gelatinolytic activity of Mock and CD9-HT1080 cells treated with DMSO or EGFR specific tyrosine kinase inhibitors gefitinib, erlotinib, lapatinib, and AG1478, respectively, was measured after 24 h on FN using gelatin zymography and quantitated after three independent repeats. Data are presented as mean ± SD (*$p < 0.05$, **$p < 0.05$). (E) Representative images of matrigel invasion assays used to assess cellular invasion in Mock and CD9-HT1080 cells treated with DMSO or gefitinib after 20 h. Cells that invaded through matrigel were fixed and stained with crystal violet. Quantitation of the number of invasive cells per field of view is presented in the bar graph below (**$p < 0.05$).

EGFR signaling using EGFR TKIs negatively regulated the increase in pro-MMP-9 release observed in CD9-HT1080 cells, we explored if silencing EGFR affected pro-MMP-9 levels. We used siRNA directed against EGFR to knock down EGFR expression in CD9-HT1080 cells and a scrambled (Ctr) siRNA for a control treatment. The mRNA and protein levels of CD9 were not affected by Ctr or EGFR siRNA treatment. However, total EGFR mRNA levels were significantly decreased in the EGFR siRNA treated CD9-HT1080 cells and were not affected upon treatment with Ctr siRNA (Fig. 3A). MMP-9 mRNA levels were also significantly decreased in the EGFR siRNA treated CD9-HT1080 cells compared to the Ctr siRNA treated CD9-HT1080 cells (Fig. 3A). The protein levels of CD9 in the CD9-HT1080 cells were unaffected by treatment with EGFR siRNA (Fig. 3B). Total EGFR levels were similar between the Mock and CD9-HT1080 cells treated with Ctr siRNA, but were less in CD9-HT1080 cells treated with EGFR siRNA. Because CD9 and EGFR were demonstrated to co-immunoprecipate from HT1080 cells [16], we accessed whether any changes in the cell surface expression of CD9 occurred upon knockdown of EGFR. Flow cytometry was used to assess CD9 cell surface expression levels upon treatment with Ctr and EGFR siRNA in both Mock and CD9-HT1080 cells. There were no significant differences in CD9 expression as a consequence of EGFR knockdown in CD9-HT1080 cells (Fig. 3C). Therefore, we attribute any phenotypic changes to total EGFR expression levels and not to changes in CD9 expression or localization.

Pro-MMP-9 release into the media and cell invasion were assessed after treatment with Ctr and EGFR siRNA using gelatin zymography and a matrigel invasion assay, respectively. Upon knockdown of EGFR, there were significantly decreased levels of pro-MMP-9 released into the media of cells as demonstrated by gelatin degradation (Fig. 3D). Furthermore, the CD9-HT1080 cells lacking EGFR were significantly less invasive than their Ctr siRNA treated CD9-HT1080 cells (Fig. 3E). These results indicated that the absence EGFR in CD9-HT1080 cells was sufficient to decrease MMP-9 mRNA and protein levels, and this absence reduced CD9-HT1080 invasion without affecting the mRNA, protein, or cell surface levels of CD9.

4. Discussion

It is well-established that EGFR activation results in increased MMP-9 expression and release [20,22] and that MMP-9 expression results in increased cell invasion [23,24]. However, the mechanism for how CD9 may regulate MMP-9 expression is not entirely clear. There are indications that CD9 directly associates with and downregulates EGFR signaling by facilitating the rate of receptor internalization after ligand binding [13]. This finding was confused by the fact that an inhibitory antibody to CD9, ALB6, and CD9 overexpression had the same outcome [13]. Furthermore, another group demonstrated that CD9 did not associate with EGFR, but regulated EGFR juxtacrine signaling by associating with the transmembrane EGFR ligand TGFα [12]. This report showed that CD9 regulated the release of pro-MMP-9 in HT1080 cells via EGFR expression and activity. We confirmed this finding and now are the first to demonstrate conclusively that EGFR activity is necessary for the augmented pro-MMP-9 released and regulated by CD9 expression using four distinct EGFR tyrosine kinase inhibitors. All inhibitors demonstrated a dose-dependent inhibition of pro-MMP-9 indicating their specificity. Treatment with 10 μM gefitinib inhibited pro-MMP-9 release to a level comparable to Mock-HT1080 cells. Consequently, we demonstrated same concentration of gefitinib resulted in the suppression of CD9-HT1080 cell invasion to the level of Mock-HT1080 cells. Therefore, inhibition of EGFR activity is sufficient

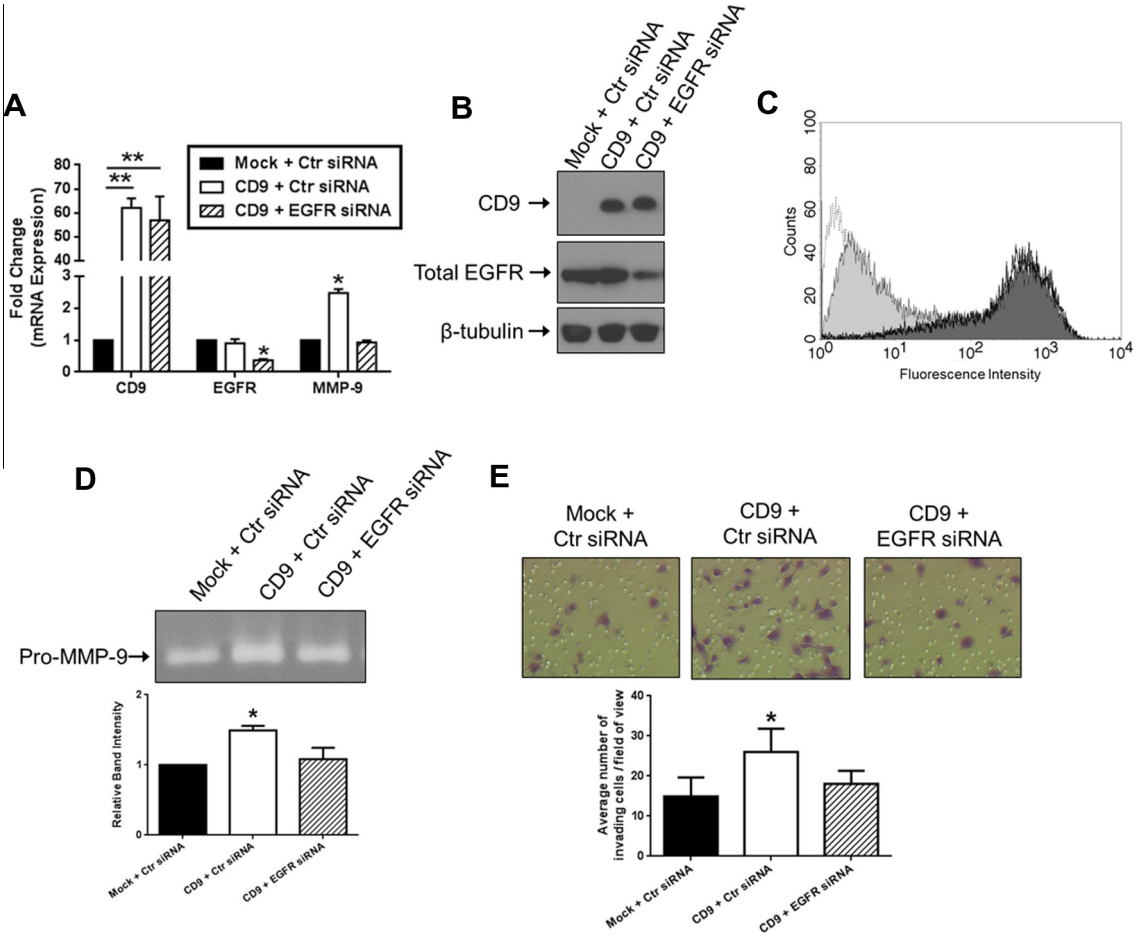

Fig. 3. Knockdown of EGFR results in decreased pro-MMP-9 expression and release without affecting the expression or localization of CD9. (A) The fold change in mRNA expression of CD9, EGFR, and MMP-9 in Mock- and CD9-HT1080 cells treated with Ctr or EGFR siRNA was measured using qRT-PCR. (B) Protein expression of CD9 and total EGFR expression was measured using Western blot analysis of Mock- and CD9-HT1080 cell lysates treated with Ctr or EGFR siRNA. (C) Flow cytometric analysis of nonpermeabilized Mock- and CD9-HT1080 cells for CD9 expression after treatment with Ctr or EGFR siRNA. Dotted line represents non-specific mIgG binding, light-gray shaded histogram is mAb7 (anti-CD9) staining of Mock + Ctr siRNA, dark gray shaded histogram is mAb7 staining of CD9 + Ctr siRNA, and the solid line unshaded histogram is mAb7 staining of CD9 + EGFR siRNA. (D) Pro-MMP-9 release into the cell media after treatment with Ctr or EGFR siRNA was assessed using gelatin zymography. (E) Matrigel invasion assay of Mock- and CD9-HT1080 cells after treatment with Ctr or EGFR siRNA and quantification of the average number of cells that invaded per field of view. Bar graphs represent mean ± SD ($^{*}p < 0.01$, $^{**}p < 0.05$).

to inhibit pro-MMP-9 release which results in decreased cellular invasion in CD9-HT1080 cells.

The expression of total EGFR in Mock, CD9, and Δ6 HT1080 cells was not significantly different (Fig. 3B; unpublished observations). Therefore, the regulation of the EGFR signaling is either at the cell surface level of the receptor or at the level of EGFR phosphorylation. Our experimental design of plating the cells on fibronectin in serum-free media was similar to other studies that demonstrated ECM-mediated activation of EGFR in the absence of EGFR ligands [25]. We have established that CD9 associates with integrin α5β1 in multiple cell lines and that it plays a role in regulating the activation state of β1 integrins [26,27]. Therefore, we hypothesize full-length CD9 expression may stabilize CD9-α5β1 interactions with fibronectin and augment intergrin-mediated EGFR activation to stimulate increased pro-MMP-9 release. Consequently, in the absence of CD9 (Mock-HT1080 cells) or when CD9 is not associated with α5β1 integrin (Δ6-HT1080 cells), pro-MMP-9 expression and release or cell invasion is not increased over baseline invasion [4]. The same results are observed when we inhibit EGFR activation using EGFR tyrosine kinase inhibitors or decrease the expression of EGFR. Consequently, we propose that CD9 regulates EGFR signaling in HT1080 cells by a CD9-α5β1-FN interaction that stimulates EGFR phosphorylation.

In conclusion, our results demonstrate that the activation of EGFR is necessary for the increase in both pro-MMP-9 release and migration observed in CD9-HT1080 cells. Although EGFR inhibitors have previously been implicated to decrease pro-MMP-9 expression and release, this is the first study to use multiple EGFR specific inhibitors to demonstrate this effect in a dose-dependent manner. Additionally, we confirmed that the expression of EGFR was necessary for pro-MMP-9 expression and release and subsequent cell invasion. This finding has clinical implications as MMP-9 and EGFR coexpression was positively correlated with a poor prognosis in human non-small cell lung cancer (NSCLC) biopsies [17]. We suggest that screening for CD9 expression in conjunction with MMP-9 and EGFR in NSCLC biopsies may significantly improve the prognostic value as CD9 expression has also been implicated in NSCLC malignancies [28,29].

Acknowledgments

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