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Matrix Metalloproteinase 14 promotes lung cancer by cleavage of Heparin-Binding EGF-like Growth Factor[1,2]

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Abstract

Molecularly targeted therapies benefit approximately 15–20% of non-small cell lung cancer (NSCLC) patients carrying specific drug-sensitive mutations. Thus, there is a clinically unmet need for the identification of novel targets for drug development. Here, we performed RNA-deep sequencing to identify altered gene expression between malignant and non-malignant lung tissue. Matrix Metalloproteinase 14 (MMP14), a membrane-bound proteinase, was significantly up-regulated in the tumor epithelial cells and intratumoral myeloid compartments in both mouse and human NSCLC. Overexpression of a soluble dominant negative MMP14 (DN-MMP14) or pharmacological inhibition of MMP14 blocked invasion of lung cancer cells through a collagen I matrix *in vitro* and reduced tumor incidence in an orthotopic K-Ras$^{G12D/+}$p53$^{-/-}$ mouse model of lung cancer. Additionally, MMP14 activity mediated proteolytic processing and activation of Heparin-Binding EGF-like Growth Factor (HB-EGF), stimulating the EGFR signaling pathway to increase proliferation and tumor growth. This study highlights the potential for development of therapeutic strategies that target MMP14 in NSCLC with particular focus on MMP14-HB-EGF axis.

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Introduction

Lung cancer remains the leading cause of cancer-related death, resulting in 1.4 million deaths annually worldwide [1]. Non-small-cell lung carcinoma (NSCLC) accounts for 80% of all lung cancers [2]. Despite advances in treatment options prognosis for NSCLC patients remains dismal. Therefore, further molecular analysis of NSCLC is necessary for the development of additional novel and specific targeted therapies for NSCLC.

Matrix Metalloproteinases (MMPs) comprise a family of proteolytic enzymes involved in the degradation of extracellular matrix (ECM) [3]. Of the various MMPs, MMP14, 15, 16, 23, and 24 are the membrane bound. MMP14 (MT1-MMP) is unique as it is the only membrane-bound MMP capable of degrading collagen I, therefore playing a crucial role in cellular migration through ECM. Notably, MMP14 null mice develop abnormalities and die by 4 weeks, suggesting that MMP14 deficiency cannot be compensated by other MMPs [4]. MMP14 is upregulated in many human tumors [5,6] and elevated levels of MMP14 and its substrate MMP2, correlate with poor prognosis and increased metastasis [5].

Activation of MMP14 molecule is regulated by the hemopexin domain (HPX) located between the catalytic domain (Cat) of the enzyme and its transmembrane fragment (TM). HPX domain is involved in recognition of proteolytic substrates of MMP14 and dimerization of HPX significantly increases activity of MMP14, which cleaves and activates pro-MMP2 and pro-MMP13 [7]. This further promotes ECM proteolysis [8,9], and results in enhanced migration, invasion and metastatic dissemination of tumor cells [10,11]. MMP14 localizes at the leading edge of invadopodia in migrating cells *via* its interaction with glycoprotein receptor CD44 [12,13]. Interaction with CD44 receptor is suggested to induce phosphorylation of the EGF receptor, and downstream activation of the MAPK and PI3K signaling pathways [14].

Clinical data shows that MMP14 expression is increased in NSCLC compared to normal lung tissue, and MMP14 is associated with poor prognosis [15]. Notably, in lung regeneration, endothelial MMP14 cleaves heparin bound (HB)-EGF, a member of the epidermal growth factor (EGF) family, and the bioavailable EGF activates cell proliferation *via* the EGFR pathway. However, the functional contribution of MMP14 in NSCLC remains poorly understood, and the potential of MMP14 inhibition has not been explored. In this study, we show that MMP14 expression is upregulated in both the epithelial and myeloid compartments of the tumor microenvironment in patients, and in an orthotopic mouse model of NSCLC. Furthermore, we provide mechanistic insights by which MMP14 contributes to NSCLC progression, and demonstrate that blocking the proteolytic activity of MMP14 can effectively block tumor growth.

Materials and Methods

Mouse Model

All animal work was conducted in accordance with a protocol approved by the Institutional ACUC at WCMC. The HKP1 lung cancer cells was derived from KP tumor lungs [16], and was cultured in DMEM with 10% FBS. 1x10 [5] of HKP1 cells were administered *via* tail vein to C57BL/6 mice (Jackson Laboratory) to generate orthotopic lung cancer and imaged by bioluminescence imaging (BLI) system (IVIS, Caliper Life Sciences).

Tissue Microarrays (TMAs)

For evaluation of MMP14 expression in NSCLC patients we used TMAs derived from 210 lung cancer patients from WCMC. Seventy-four percent of the patients were at stage IA/IB, 8% at stages IIA/IIB, 12% at stage III and 6% at stage IV. Immunohistochemical staining of MMP14 (Clone LEM-2/63.1, Abcam) was performed using the Bond III Autostainer (Leica Microsystems, IL, USA). TMAs were examined in a double blinded manner by two individuals using scale 0 to 3 with score >1 considered positive.

Generation of KP Cell Lines Expressing Dominant Negative FLAG-MMP14

Mouse MMP14 cDNA (Open Biosystems) was cloned into pCDH-EF1-MCS-T2A-copGFP lentiviral vector (System Biosciences). Analogically we generated a dominant negative (DN) form of MMP14-GFP (ΔTMΔCat MMP14) containing deletions between Tyr112-Gly288 and Ala536-Val582 [17]. For detection, a FLAG tag was inserted between Arg111 and Tyr112. Lentiviral particles were produced in 293 T cells by co-transfection of the MMP14 construct with Lenti Packaging Mix (Advanced Biomedical Materials). GFP positive cells were selected by FACS sorting (LSRII, BD) 5 days after infection. As a positive control for MMP14 inhibition we used NSC405020 small molecule inhibitor at 50uM concentration (Tocris).

Results

MMP14 is Upregulated in Epithelial and Myeloid Compartments in Mouse NSCLC

Using an orthotopic Kras-driven NSCLC mouse model (*K-ras*$^{LSL\text{-}G12D/+}$;*p53* $^{flox/flox}$), we have evaluated the differential gene expression in epithelial and intratumoral stromal cells by RNA-seq analysis [18]. Interestingly, we found that MMP14 was significantly upregulated both in intratumoral myeloid cells (CD11b + F4/80+ macrophages, Ly6C+ monocytes, Ly6G+ neutrophils) and EpCam + tumor cells (Figure 1*A* and Supplementary Table 1). Quantitative RT-PCR analysis (Figure 1*B*) and Western-blot (Figure 1*C*) confirmed increased expression of MMP14 in the intratumoral myeloid and epithelial compartments. Immunofluorescence staining of lung sections showed that membrane-specific MMP14 expression was detected in both the epithelial (EpCam+) cells and hematopoietic (CD45+) stromal cells in the tumors (Figure 1*D*).

Together, these data show that MMP14 expression is markedly increased in NSCLC tumor, which was contributed by both tumor epithelial and stromal cells.

MMP14 is Up-Regulated in Epithelial and Myeloid Compartments in Human NSCLC

The up-regulation of MMP14 expression in tumor tissue was also observed in NSCLC patients. We have performed RNA-seq analysis of individual cell populations isolated from patient-derived tumors compared to matched adjacent non-neoplastic lung tissue [19]. The result showed that MMP14 was specifically upregulated in tumor tissue compared to adjacent lungs (Figure 2*A* and Supplementary Table 2) with MMP14 expression increased in both intratumoral myeloid cells (CD11b + CD33+ monocytes, CD11b + CD33- neutrophils) and EpCam + epithelial cells (Figure 2*A*). The increase expression of MMP14 was confirmed by qRT-PCR (Figure 2*B*). Consistently, immunofluorescence staining showed that MMP14 expression was found in both epithelial (EpCam+) cells and hematopoietic (CD45+) cells in the tumors (Figure 2*C*). With a tissue microarray (TMA) derived from a large cohort of NSCLC patients (n = 210), high MMP14 expression was detected in approximately 68% of patients. Among them, 10% showed

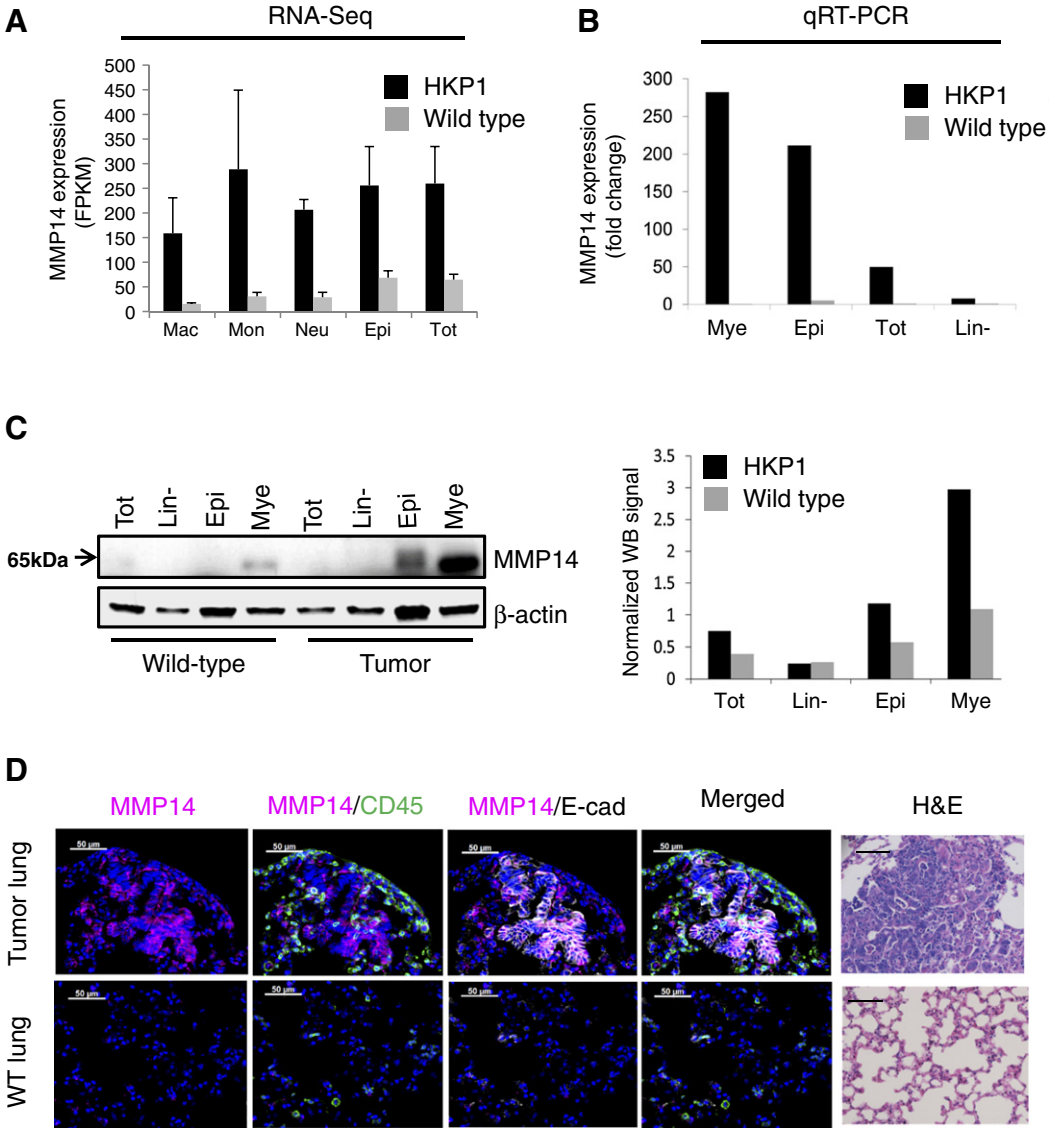

Figure 1. MMP14 is differentially expressed in epithelial and stromal cell in K-Ras$^{+/G12D}$p53$^{-/-}$(HKP1) mouse model for NSCLC. (A) RNA sequencing analysis of MMP14 gene expression in macrophage (CD11b+, F4/80+), monocytes (Ly6C+), neutrophil (Ly6G+) and epithelial (EpCam+) cells in mice with HKP1 lung tumors as compared to wild-type lungs. Tot, Total lung cells. Error bars depict standard deviation. (B) qRT-PCR analysis showing MMP14 gene expression in myeloid (CD11b + CD11c+), epithelial (EpCam+), total lung cells (Tot) and lineage negative (Lin-) cell populations. (C) Upper panel, Western blot analysis of MMP14 protein levels in epithelial (EpCam+) and myeloid (CD11b+) populations in lung tumors compared to wild-type lungs. Lower panel, Quantification of the western blot showing MMP14 protein levels normalized to actin. Tot, total cells; Lin-, lineage negative; Epi, epithelial cells; Mye, myeloid cells. (D). Immunofluorescence staining of epithelial (E-cadherin+) and hematopoietic (CD45+) cell population-expressing MMP14 in HKP1 tumors.

MMP14 staining in both stromal and epithelial compartments (Figure 2, *D* and *E*).

The consistent high expression of MMP14 in both mouse lung cancer model and NSCLC patients suggests that MMP14 may play a pivotal role in NSCLC progression.

Dominant Negative MMP14 Inhibits Invasion of Cancer Cells by Suppressing Their Ability to Degrade Collagen I

To obtain insights by which MMP14 contributes to NSCLC, we sought to generate MMP14 loss-of-function in HKP1 lung tumor cells. Given that homodimerization of MMP14 through the hemopexin (HPX) domain is important for its optimal proteolytic activity [17,20], we chose to use to a dominant negative (DN) approach. The DN-MMP14 (ΔTMΔCat-FLAG-MMP14) construct lacks the transmembrane and the catalytic domains (Figure 3*A*). Since the hemopexin (HPX) domain remains intact, the DN-MMP14 is still able to dimerize with wild type MMP14 and block its catalytic function [7] probably by inhibiting ability of MMP14 to bind collagen.

HKP1 cells overexpressing the DN-MMP14 were generated by infecting wild type HKP1 cells with lentivirus carrying DN-MMP14. Western blot analysis of HKP1 cell lysates showed robust expression of DN-MMP14 (40 kDa) as well as DN-MMP14-GFP fusion protein (64 kDa), possibly due to imperfect efficiency of ribosome

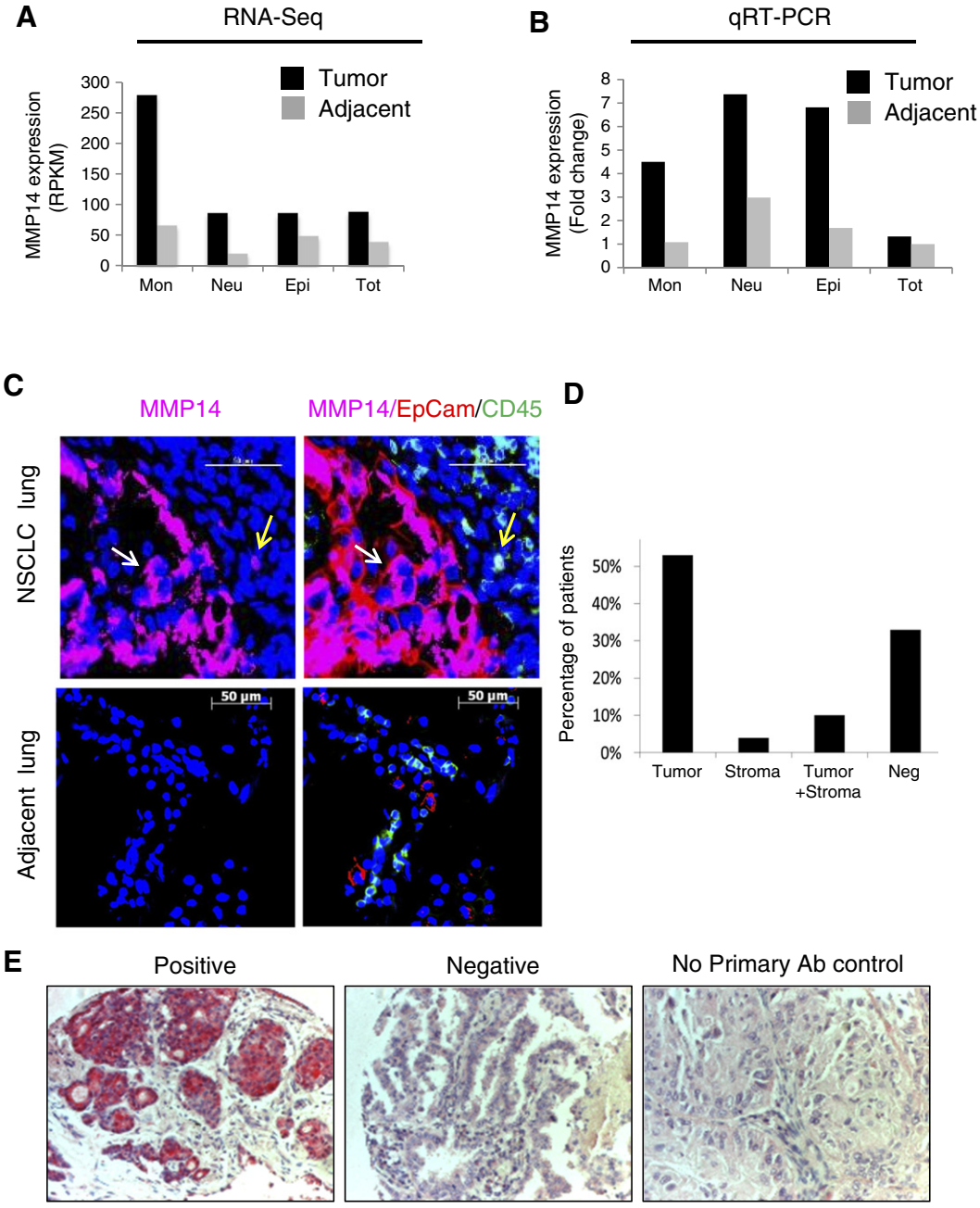

Figure 2. MMP14 is differentially expressed in the epithelial and stromal cells in NSCLC patients. (A) RNA sequencing analysis of MMP14 gene expression in the monocytes (CD11b + CD33+), neutrophils (CD11b + CD33-) and epithelial cells (EpCam+) derived from human NSCLC as compared to the non-neoplastic adjacent lung tissue. Tot, total lung cells. (B) qRT-PCR showing MMP14 expression in cell types depicted in panel A. (C) Representative immunofluorescence images of MMP14 expression in the epithelial (EpCam+, white arrows) and bone marrow-derived stromal (CD45+, yellow arrows) cells in tumor and matched adjacent non-neoplastic lung in adenocarcinoma patients. (D) Percentage of patients showing MMP14 expression in the tumor, stroma or both as determined by tissue microarray (TMA) analysis. (E) IHC staining of MMP14 of the lung tumor tissue array showing the positive (left), the negative (middle) and no primary antibody control (right).

skipping. Of note, both DN-MMP14 (40 kDa) and DN-MMP14-GFP (64 kDa) were efficiently secreted, as both bands were detected in the supernatant compartment (Figure 3*B*). The overexpression of DN-MMP14 did not affect the expression of endogenous MMP14 (64–66 kDa) (Figure 3*B*). The surface presentation of endogenous MMP14 was not affected either as confirmed by surface biotinylation assay (Figure 3*C*). Interestingly, DN-MMP14 expressing cells displayed decreased levels of active MMP-2, suggesting that dominant-negative approach blocks effectively MMP14-mediated processing of MMP2 precursor (Figure S3).

Comparison of proliferation rates showed that both control (expressing GFP) and DN-MMP14 expressing HKP1 cells proliferated at similar rate. Interestingly in a serum free media control HKP1 cells retained high proliferation while the proliferation of DN-MMP14 cells was significantly impaired (Supplementary Figure S1). Such observation suggested that MMP14 through its proteolytic

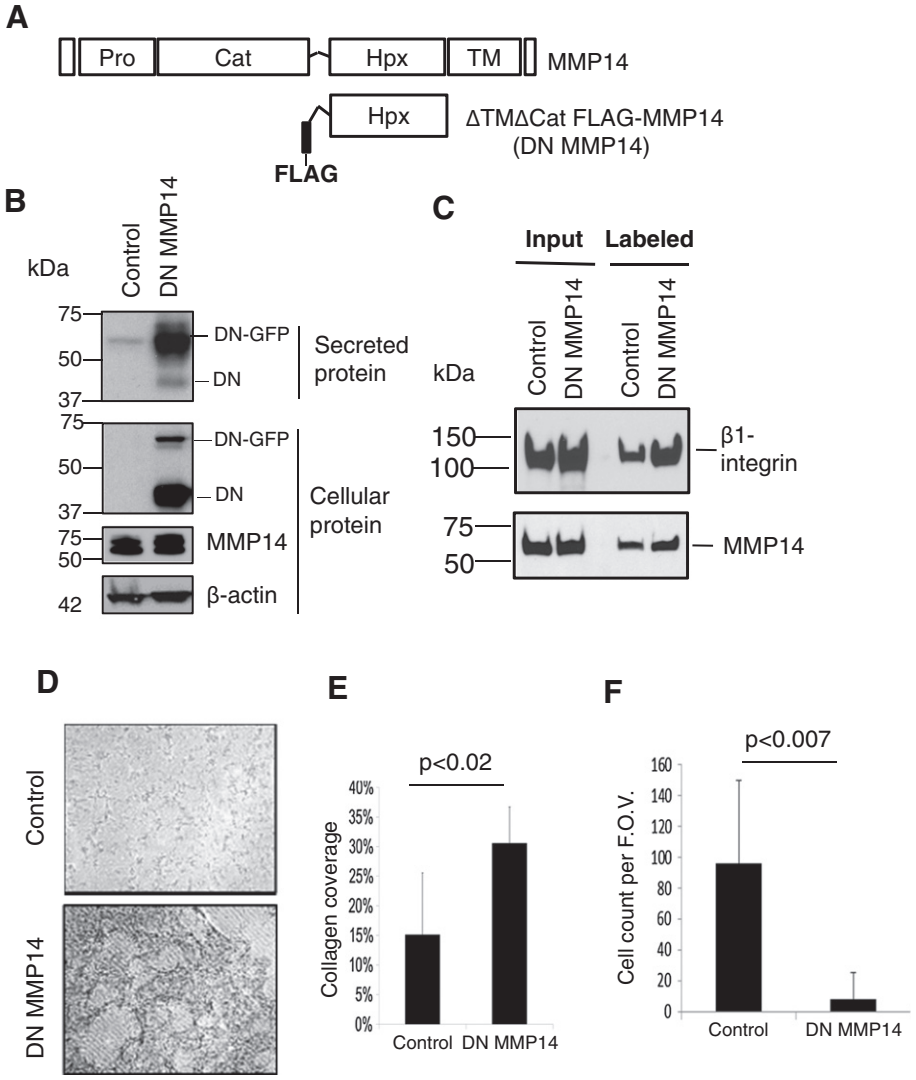

Figure 3. HKP1 cells expressing dominant negative MMP14 are defective in the degradation and invasion through type I collagen *in vitro.* (A) Schematic representation of full length MMP14 and truncated soluble dominant negative ΔTM ΔCat FLAG-MMP14 (DN-MMP14). Pro (Pro domain), Cat (Catalytic domain), Hpx (Hemopexin domain), TM (Transmembrane domain). MMP14 constructs were cloned in pCDH-EF1-MCS-T2A-copGFP lentiviral vector, in which GFP reporter is preceded by a T2A peptide. (B) Western blot analysis of MMP14 and DN-MMP14 in the cellular and secreted fraction of control and HKP1 cells. "DN-GFP" denotes DN-MMP14 fused to GFP and "DN" denotes DN-MMP14 free of GFP. DN-MMP14 was detected with anti-FLAG antibody. (C) Western blot analysis of MMP14 following surface biotinylation assay in vector control (Control) and DN-MMP14 expressing HKP1cells. β1-integrin (a cell surface protein) was used as internal control for biotin labeling and pulldown. (D) Representative images of collagen I degradation by wild type HKP1 cells (control) and DN-MMP14 HKP1 cells. (E) Quantification of collagen I degradation from panel C. Degradation was quantified as percentage of well area covered by the collagen after 72 hr. Images of 4 different wells were analyzed and p values were calculated using Student's t-test (F) Transwell migration assay of wild type HKP1 cells and DN -MMP14 expressing HKP1 cells through type I collagen-coated membrane. Cells were DAPI stained and counted per field of view (F.O.V.) 5 images. Data represents the average number of cells per FOV. Error bars denote Standard deviation. Student's t test was used for statistical analysis.

activity can help releasing growth factors that can act in an autocrine manner. Given MMP14 [21,22] is the only membrane MMP able to degrade collagen I, we tested the impact of DN-MMP14 on MMP14 function by collagen I degradation assay. Control (expressing GFP) or DN-MMP14 expressing HKP1 cells were seeded in 6-well plates coated with collagen I and cultured for 72 hr. While the control cells displayed considerable collagen degradation, the DN-MMP14 expressing cells were significantly defective in removing the collagen (Figure 3, *D* and *E*). Consistently, the DN-MMP14 expressing HKP1 cells were dysfunctional to invade through the collagen I layer in a transwell assay. They showed a 10-fold decrease in invasion as compared the control cells (Figure 3*F*). Pharmacological inhibition of MMP14 with a specific small-molecule inhibitor NS405020 decreased invasion even more severely almost totally blocking ability of HKP to migrate through collagen (Supplementary Figure S2).

Together, these results suggest that DN-MMP14 blocks MMP14 function and abrogates tumor cell invasion through the ECM possibly due to its impaired ability to degrade collagen I, which encouraged us to further investigate MMP14 roles in NSCLC *in vivo*.

DN-MMP14 Impairs Tumor Growth in the HKP1 Mouse Model of Lung Cancer

To assess the role of MMP14 *in vivo*, we employed orthotopic lung tumor model by injecting control or DN-MMP14 expressing HKP1 cells through tail vein into the syngeneic immunocompetent mice. The control HKP1 cells generated massive lung tumor burden in mice as manifested by measuring the lung weight 4 weeks after inoculation. Significantly, the expression of DN-MMP14 led to less lung tumor burden (Figure 4*A*). HC analysis of lung sections also revealed significantly bigger tumor area in control mice (18% ± 0.7), as compared to the DN-MMP14 group (0. 01% ± 0.5) (Figure 4*B*). The whole lung images showed massive hemorrhagic tumors in the control lungs, whereas tumors were not visible in lungs with DN-MMP14 HKP1 cells (Figure 4*B*).

The dynamic monitoring of tumor growth by BLI also revealed that control HKP1 cells rapidly formed tumors, whereas HKP1 cells expressing DN-MMP14 failed to generate tumors (Figure 4*C*). Further analysis of lung lysates showed that DN-MMP14 expressing tumor cells were present in the lungs at the time of sacrifice (Figure 4*D*), suggesting that DN-MMP14 cells survived in the lung, but fail to establish tumors. H&E staining of lung tissues showed a lack of detectable tumor nodules in DN-MMP14 mice, whereas large nodules were observed in the control group (Figure 4*E*, upper panel). The immunostainings consistently showed that the residual DN-MMP14 cells expressed the FLAG reporter (Figure 4*E*, lower panel). These results demonstrate that MMP14 loss-of-function results in impaired lung tumor growth *in vivo*.

MMP14 Cleaves Heparin-Binding EGF-Like Growth Factor to Activate the Protumorigenic EGFR Pathway

MMP14 is best known to accelerate tumor progression by cleavage of collagen I and degradation of ECM. Interestingly, MMP14 was recently reported to participate in the lung regeneration by activation of HB-EGF and stimulation of growth of lung epithelial cells [24]. To determine the whether the same mechanism can be used by MMP14-expressing cancer cells to promote growth of lung tumors, we re-analyzed our RNA sequencing data to identify known substrates of MMP14. HB-EGF, an important factor in survival signaling [23] and lung regeneration [24], was upregulated in HKP1 tumor epithelial cells, as well as monocytic and neutrophil populations within the tumor microenvironment (Figure 5*A*). MMP14 is known to cleave off 20 amino acids at the N-terminus of HB-EGF to generate heparin-independent growth factors: mHB-EGF (membrane-bound) and sHB-EGF (soluble) (Figure 5*B*) [25,26].

To evaluate processing of HB-EGF in the presence and absence of functional MMP14, we analyzed both the intracellular and extracellular fractions of HKP1 cells by Western blot. Control HKP1 cells expressed a 23 kDa unprocessed HB-EGF isoform in the cellular fraction, while the extracellular fraction expressed a distinct pattern of multiple isoforms (Figure 5*C*). Although DN-MMP14 HKP1 cells also expressed the 23 kDa unprocessed HB-EGF isoform in the cellular fraction, there was a marked decrease in the ~18 kDa and the ~9 kDa isoforms from the extracellular fraction (Figure 5*C*). Cleavage patterns of HB-EGF revealed that the 18 kDa and 9 kDa isoforms correspond to membrane-bound and soluble active HB-EGF respectively, consistent with previous observations [25,26]. In accordance with the HB-EGF cleavage patterns, DN-MMP14-expressing HKP1 cells showed impaired EGFR signaling, as determined by reduction in the phosphorylated EGF receptor (p-EGFR) and its downstream signaling components including ERK1/2 (Figure 5*D* and Supplementary Figure S5*A*). Consistent with our *in vitro* data, HKP1 tumors also exhibited impaired EGFR signaling (Figure 5*E* and Supplementary Figure S5*B*). Immunofluorescence staining of HKP1 tumors showed increased p-EGFR in the GFP+ nodules indicating that the EGFR pathway was activated in these tumors (Figure 5*F*). Together, these results suggest that MMP14-mediated cleavage of HB-EGF, results in the activation of the protumorigenic EGFR pathway in NSCLC.

Discussion

Clinical studies have shown that MMP14 expression is elevated in NSCLC cells, and confers poor prognosis in NSCLC patients [14,15]. However, the contribution of MMP14 in NSCLC has not been widely studied, and the therapeutic potential of MMP14 inhibition remains unexplored. By analyzing specific cellular compartments in tumor tissue, we have demonstrated that MMP14 expression is elevated in both tumor epithelial cells and myeloid cells of mouse and human NSCLC.

Our study involved use of dominant negative MMP14 (DN-MMP14) that lacked the transmembrane and catalytic domains of the mature enzyme but contained intact hemopexin domain. Such construct has been reported to inhibit activity of MMP14 by dimerizing with wild-type enzyme [7]. In our study HKP1 lung cancer cells expressing DN-MMP14 were impaired in their ability to degrade collagen I, associated with decreased invasiveness and marked suppression of tumor growth. While most of the synthesized DN-MMP14 appeared to dimerize with the wild-type MMP14 on the surface of the lung tumor cells in an autocrine fashion, the secreted form in the extracellular milieu could also effectively target MMP14 on the stromal cells, thus inhibiting role of stroma-derived MMP14 on tumor progression.

MMP14 promotes metastatic dissemination of cancer cells by cleaving components of ECM, including type I and III collagen, fibronectin I and II and laminin 1 and 5 [9,10]. Following dimerization, MMP14 cleaves and activates proMMP2 and proMMP13, which enhances ECM proteolysis [7,8]. In our study, HKP1 cells expressing DN-MMP14 displayed reduced levels of active MMP2 and exhibited reduced invasion through collagen I layer, suggesting that MMP14 loss-of-function impacts tumor cell invasion through the ECM. Moreover, cells expressing DN-MMP14 were impaired in their ability to degrade collagen I, suggesting that the presence of active MMP14 may be essential for effective ECM remodeling. Furthermore, pharmacological blockade of MMP14 activity also inhibited invasion through collagen I confirming specificity of the DN-MMP14 approach. Interestingly, although our HKP1 DN-MMP14 cells did not contain deficiencies in other metalloproteinases, including ADAM10 (Supplementary Figure S4), specific inhibition of MMP14 was sufficient to produce such an overt phenotype. These observations underline the importance of MMP14 in NSCLC progression and make it an attractive therapeutic target.

Our results suggest that besides collagen degradation and ECM remodeling MMP14 can also contribute to lung cancer progression by releasing HB-EGF and activating EGFR signaling. The membrane-bound HB-EGF is not only a precursor of the soluble form, but also independently activates signaling pathways [27,28] being a potent factor in survival signaling [23]. An important co-factor of HB-EGF is heparin, which is required to unmask its

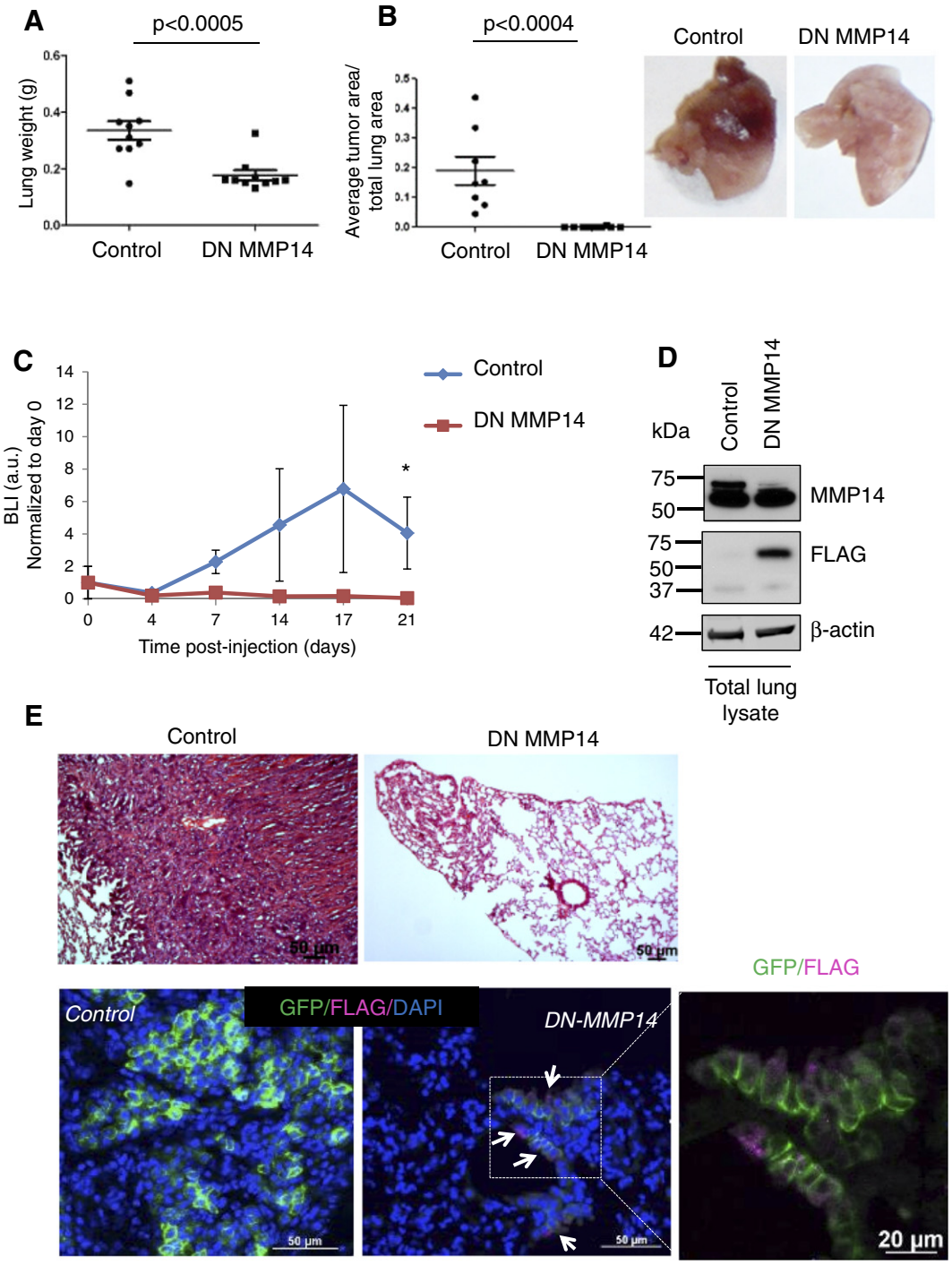

Figure 4. Dominant negative MMP14-expressing HKP1 cells fail to grow tumors *in vivo*. (A) Lung weight of C57BL/6 mice 26 days after administration of HKP1 cells expressing vector alone (vector) or DN-MMP14. (n = 10 mice per group). Error bars show SEM. (B) Lungs in panel A were stained with H&E and average tumor area in the lungs measured. Quantitation performed from 8 sections per lung (n = 10 mice per group). Error bars depict SEM.Inserts on the right show representative images of whole lung mounts. (C) BLI depicting tumor growth in mouse lungs administered with HKP1 cells expressing vector alone (vector) or DN-MMP14. Tumor growth was monitored for 21 days (n = 8 mice per group). Error bars depict SEM. * $P < .05$. (D) Western blot analysis of total lung lysate with anti-MMP14 and anti-FLAG antibody. (E) Representative H&E images of the lungs from HKP1 tumors expressing vector alone (vector) or DN-MMP14 (Top panel), and representative immunofluorescence images showing GFP+ HKP1 (vector) tumors and FLAG+ GFP+ cells in DN-MMP14 expressing HKP1 tumors in the lungs (bottom panel). A magnified image highlighting GFP and FLAG is depicted on the right.

EGF-like domain *in vitro* [29] and heparin sulfate proteoglycans (HSPG), abundant in the ECM, have been suggested to fulfill this role *in vivo* [30]. However, MMP14 has been shown to diminish the heparin requirement, as cleavage of the N-terminal heparin-binding domain makes HB-EGF heparin-independent [24,25]. Our data suggest that NSCLC co-opts MMP14-mediated HB-EGF cleavage,

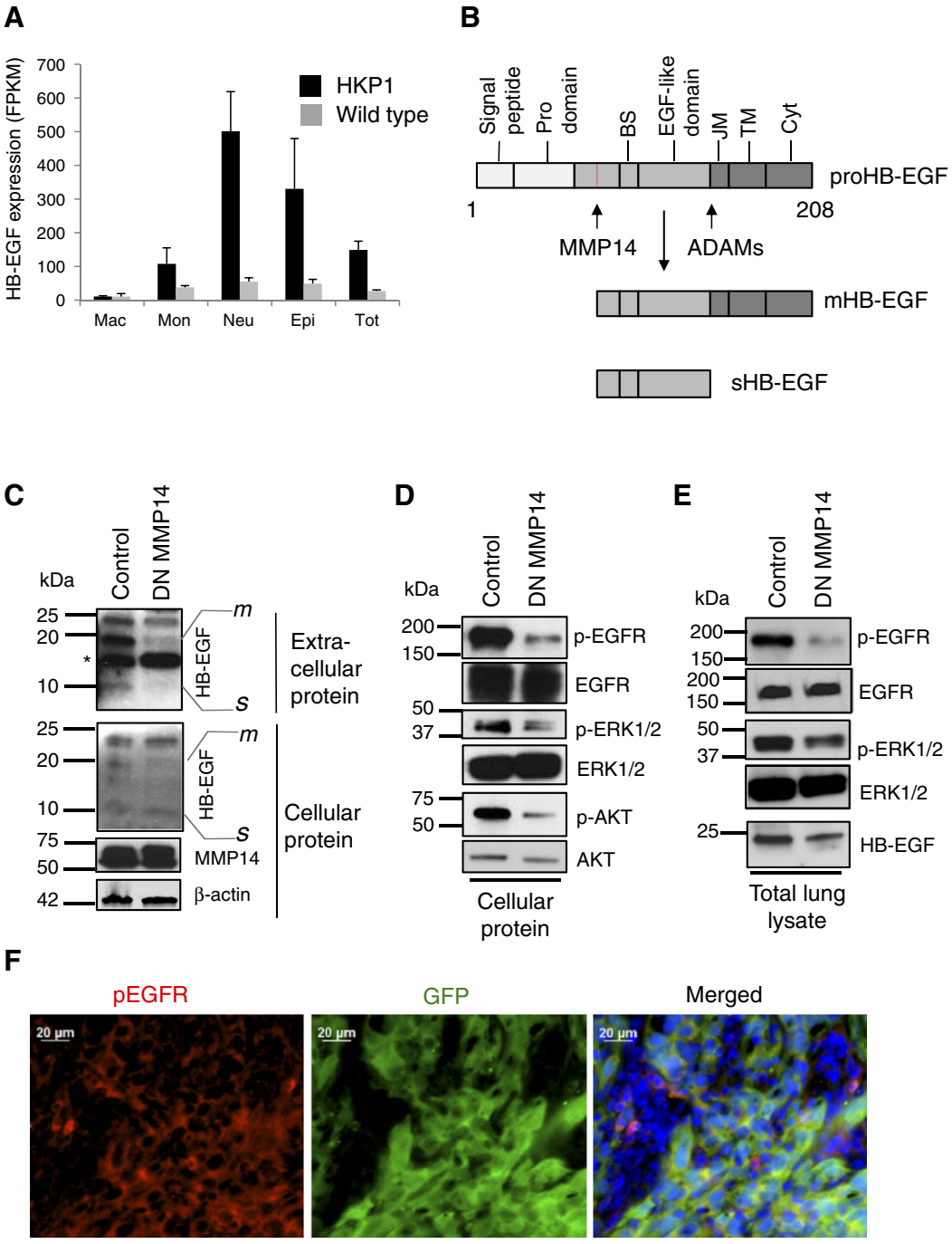

Figure 5. MMP14 activates the EGFR pathway by proteolytic processing of HB-EGF. (A) RNA sequencing analysis of HB-EGF in macrophage (CD11b + F4/80+), neutrophil (Ly6G+) and monocytes (Ly6C+) and epithelial (EpCam+) cell populations from HKP1 tumors and wild type lungs. Error bars show standard deviation. Tot, total cells. (B) Schematic representation of the cleavage patterns of HB-EGF by ADAMs and MMPs. MMP14 cleavage between residues ^{82}Ala and ^{83}Leu unmasks the EGF-like domain to generate membrane HB-EGF (mHB-EGF). ADAMs cleavage generates a soluble HG-EGF (sHB-EGF). BS, Basic Stretch; JM,Juxtamembrane domain; TM, Transmembrane domain; Cyt, Cytoplasmic tail; mHB-EGF, membrane-bound MMP14-activated HB-EGF; sHB-EGF, soluble MMP14-activated HB-EGF. (C) Western blot analysis of HB-EGF and MMP14 in lysates derived from cellular and extracellular fractions of HKP1 cells with vector alone (vector) or DN-MMP14. Membrane-bound (m) and soluble (s) forms of HB-EGF are indicated. * Denotes an unspecific band. (D) Western blot analysis of components of the EGFR pathway including p-EGFR, pERK1/2 and p-AKT in HKP1 cells *in vitro*. (E) Western blot showing components of EGFR pathway and HB-EGF in HKP1 tumors. (F) Representative immunofluorescence staining of HKP1 tumors for p-EGFR in the GFP+ tumor cells (white arrows).

rendering heparin-independent, similar to a phenomenon observed in lung regeneration [24]. The activation of HB-EGF is likely to promote proliferation and survival signaling through the EGFR pathway, as HB-EGF binds EGFR with higher affinity than EGF, and is therefore a potent inducer of tumor growth [31]. Consistently, our results have shown that impaired proteolytic

activation of HB-EGF by MMP14 correlates with impaired EGFR pathway activation.

Our findings that the MMP14/HB-EGF/EGFR axis is involved in NSCLC progression underscore the potential of this pathway in the development of targeted therapies. Previous studies have assessed the potential of either HB-EGF or MMP14 as therapeutic targets in many types of cancer [32–35]. One drawback of the first generation of MMP14 inhibitors was that they targeted the catalytic site of the enzyme, demonstrating affinity not only to MMP14 but also to other MMPs and non-MMP proteases. These off-targeting effects often led to non-specific and severe toxic effects. In this study, we have evaluated the approach of DN-MMP14, which targets the hemopexin domain of MMP14. This allows for selective binding and potentially eliminates off-target effects. Expression of DN-MMP14 resulted in effective inhibition of NSCLC growth, thereby suggesting that blocking the hemopexin domain comprises an attractive therapeutic strategy. Our results is consistent with the previous report showing that the synthetic peptides targeting the hemopexin domain of MMP14 inhibited MMP-14-dependent cell migration, slowed growth of breast tumors, and did not impact other MMPs [13].

Altogether, our study suggests that MMP14 plays an important mechanistic role in NSCLC progression, by supporting cancer invasiveness, promoting collagen degradation, and releasing HB-EGF, which accelerates lung tumor progression. Inhibiting the MMP14- through targeting hemopexin domain of MMP14 may constitute a novel approach in lung cancer therapy that would significantly improve therapeutic outcome in NSCLC patients.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2016.11.005.

References

[1] Siegel R, Ma J, Zou Z, and Jemal A (2014). Cancer statistics, 2014. *CA Cancer J Clin* **64**(1), 9–29.

[2] Pao W and Hutchinson KE (2012). Chipping away at the lung cancer genome. *Nat Med* **18**(3), 349–351.

[3] Egeblad M and Werb Z (2002). New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* **2**(3), 161–174.

[4] Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, Mankani M, Robey PG, Poole AR, and Pidoux I, et al (1999). MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell* **99**(1), 81–92.

[5] Tetu B, Brisson J, Wang CS, Lapointe H, Beaudry G, Blanchette C, and Trudel D (2006). The influence of MMP-14, TIMP-2 and MMP-2 expression on breast cancer prognosis. *Breast Cancer Res* **8**(3), R28.

[6] Crispi S, Calogero RA, Santini M, Mellone P, Vincenzi B, Citro G, Vicidomini G, Fasano S, Meccariello R, and Cobellis G, et al (2009). Global gene expression profiling of human pleural mesotheliomas: identification of matrix metalloproteinase 14 (MMP-14) as potential tumour target. *PLoS One* **4**(9)e7016.

[7] Li XY, Ota I, Yana I, Sabeh F, and Weiss SJ (2008). Molecular dissection of the structural machinery underlying the tissue-invasive activity of membrane type-1 matrix metalloproteinase. *Mol Biol Cell* **19**(8), 3221–3233. http://dx.doi.org/10.1091/mbc.E08-01-0016 [Epub 2008 May 21].

[8] Itoh Y, Ito N, Nagase H, Evans RD, Bird SA, and Seiki M (2006). Cell surface collagenolysis requires homodimerization of the membrane-bound collagenase MT1-MMP. *Mol Biol Cell* **17**(12), 5390–5399.

[9] Itoh Y and Seiki M (2006). MT1-MMP: a potent modifier of pericellular microenvironment. *J Cell Physiol* **206**(1), 1–8.

[10] Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E, and Seiki M (1994). A matrix metalloproteinase expressed on the surface of invasive tumour cells. *Nature* **370**(6484), 61–65.

[11] Kessenbrock K, Plaks V, and Werb Z (2010). Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell* **141**(1), 52–67.

[12] Artym VV, Zhang Y, Seillier-Moiseiwitsch F, Yamada KM, and Mueller SC (2006). Dynamic interactions of cortactin and membrane type 1 matrix metalloproteinase at invadopodia: defining the stages of invadopodia formation and function. *Cancer Res* **66**(6), 3034–3043.

[13] Mori H, Tomari T, Koshikawa N, Kajita M, Itoh Y, Sato H, Tojo H, Yana I, and Seiki M (2002). CD44 directs membrane-type 1 matrix metalloproteinase to lamellipodia by associating with its hemopexin-like domain. *EMBO J* **21**(15), 3949–3959.

[14] Zarrabi K, Dufour A, Li J, Kuscu C, Pulkoski-Gross A, Zhi J, Hu Y, Sampson NS, Zucker S, and Cao J (2011). Inhibition of matrix metalloproteinase 14 (MMP-14)-mediated cancer cell migration. *J Biological Chem* **286**(38), 33167–33177.

[15] Wang YZ, Wu KP, Wu AB, Yang ZC, Li JM, Mo YL, Xu M, Wu B, and Yang ZX (2014). MMP-14 overexpression correlates with poor prognosis in non-small cell lung cancer. *Tumour Biol* **35**(10), 9815–9821.

[16] DuPage M, Dooley AL, and Jacks T (2009). Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. *Nat Protoc* **4**(7), 1064–1072.

[17] Nonaka T, Nishibashi K, Itoh Y, Yana I, and Seiki M (2005). Competitive disruption of the tumor-promoting function of membrane type 1 matrix metalloproteinase/matrix metalloproteinase-14 in vivo. *Mol Cancer Ther* **4**(8), 1157–1166.

[18] Choi H, Sheng J, Gao D, Li F, Durrans A, Ryu S, Lee SB, Narula N, Rafii S, and Elemento O, et al (2015). Transcriptome analysis of individual stromal cell populations identifies stroma-tumor crosstalk in mouse lung cancer model. *Cell Rep* **10**(7), 1187–1201.

[19] Durrans A, Gao D, Gupta R, Fischer KR, Choi H, El Rayes T, Ryu S, Nasar A, Spinelli CF, and Andrews W, et al (2015). Identification of Reprogrammed Myeloid Cell Transcriptomes in NSCLC. *PLoS One* **10**(6)e0129123.

[20] Cao J, Kozarekar P, Pavlaki M, Chiarelli C, Bahou WF, and Zucker S (2004). Distinct roles for the catalytic and hemopexin domains of membrane type 1-matrix metalloproteinase in substrate degradation and cell migration. *J Biol Chem* **279**(14), 14129–14139.

[21] Sabeh F, Shimizu-Hirota R, and Weiss SJ (2009). Protease-dependent versus -independent cancer cell invasion programs: three-dimensional amoeboid movement revisited. *J Cell Biol* **185**(1), 11–19.

[22] Sabeh F, Ota I, Holmbeck K, Birkedal-Hansen H, Soloway P, Balbin M, Lopez-Otin C, Shapiro S, Inada M, and Krane S, et al (2004). Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP. *J Cell Biol* **167**(4), 769–781.

[23] Singh AB, Sugimoto K, and Harris RC (2007). Juxtacrine activation of epidermal growth factor (EGF) receptor by membrane-anchored heparin-binding EGF-like growth factor protects epithelial cells from anoikis while maintaining an epithelial phenotype. *J Biol Chem* **282**(45), 32890–32901.

[24] Ding BS, Nolan DJ, Guo P, Babazadeh AO, Cao Z, Rosenwaks Z, Crystal RG, Simons M, Sato TN, and Worgall S, et al (2011). Endothelial-derived angiocrine

signals induce and sustain regenerative lung alveolarization. *Cell* **147**(3), 539–553.

[25] Koshikawa N, Mizushima H, Minegishi T, Iwamoto R, Mekada E, and Seiki M (2010). Membrane type 1-matrix metalloproteinase cleaves off the NH2-terminal portion of heparin-binding epidermal growth factor and converts it into a heparin-independent growth factor. *Cancer Res* **70**(14), 6093–6103.

[26] Koshikawa N, Mizushima H, Minegishi T, Eguchi F, Yotsumoto F, Nabeshima K, Miyamoto S, Mekada E, and Seiki M (2011). Proteolytic activation of heparin-binding EGF-like growth factor by membrane-type matrix metalloproteinase-1 in ovarian carcinoma cells. *Cancer Sci* **102**(1), 111–116.

[27] Iwamoto R and Mekada E (2000). Heparin-binding EGF-like growth factor: a juxtacrine growth factor. *Cytokine Growth Factor Rev* **11**(4), 335–344.

[28] Ongusaha PP, Kwak JC, Zwible AJ, Macip S, Higashiyama S, Taniguchi N, Fang L, and Lee SW (2004). HB-EGF is a potent inducer of tumor growth and angiogenesis. *Cancer Res* **64**(15), 5283–5290.

[29] Takazaki R, Shishido Y, Iwamoto R, and Mekada E (2004). Suppression of the biological activities of the epidermal growth factor (EGF)-like domain by the heparin-binding domain of heparin-binding EGF-like Growth Factor. *J Biol Chem* **279**(45), 47335–47343.

[30] Higashiyama S, Abraham JA, and Klagsbrun M (1993). Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: dependence on interactions with cell surface heparan sulfate. *J Cell Biol* **122**(4), 933–940.

[31] Higashiyama S, Abraham JA, Miller J, Fiddes JC, and Klagsbrun M (1991). A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* **251**(4996), 936–939.

[32] Kunami N, Yotsumoto F, Ishitsuka K, Fukami T, Odawara T, Manabe S, Ishikawa T, Tamura K, Kuroki M, and Miyamoto S (2011). Antitumor effects of CRM197, a specific inhibitor of HB-EGF, in T-cell acute lymphoblastic leukemia. *Anticancer Res* **31**(7), 2483–2488.

[33] Tsujioka H, Fukami T, Yotsumoto F, Ueda T, Hikita S, Takahashi Y, Kondo H, Kuroki M, and Miyamoto S (2011). A possible clinical adaptation of CRM197 in combination with conventional chemotherapeutic agents for ovarian cancer. *Anticancer Res* **31**(7), 2461–2465.

[34] Sanui A, Yotsumoto F, Tsujioka H, Fukami T, Horiuchi S, Shirota K, Yoshizato T, Kawarabayashi T, Kuroki M, and Miyamoto S (2010). HB-EGF inhibition in combination with various anticancer agents enhances its antitumor effects in gastric cancer. *Anticancer Res* **30**(8), 3143–3149.

[35] Devy L, Huang L, Naa L, Yanamandra N, Pieters H, Frans N, Chang E, Tao Q, Vanhove M, and Lejeune A, et al (2009). Selective inhibition of matrix metalloproteinase-14 blocks tumor growth, invasion, and angiogenesis. *Cancer Res* **69**(4), 1517–1526.