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P38 pathway as a key downstream signal of connective tissue growth factor to regulate metastatic potential in non-small-cell lung cancer

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Key words

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Although the secretory matricellular protein connective tissue growth factor (CTGF) has been reported to be related to lung cancer metastasis, the precise mechanism by which CTGF regulates lung cancer metastasis has not been elucidated. In the present study, we show the molecular link between CTGF secretion and the p38 pathway in the invasive and metastatic potential of non-small-cell lung cancer (NSCLC). Among three different human NSCLC cell lines (PC-14, A549, and PC-9), their *in vitro* invasiveness was inversely correlated with the level of CTGF secretion. By supplementing or reducing CTGF secretion in NSCLC culture, dysregulation of the invasive and metastatic potential of NSCLC cell lines was largely compensated. By focusing on the protein kinases that are known to be regulated by CTGF, we found that the p38 pathway is a key downstream signal of CTGF to regulate the metastatic potential of NSCLC. Importantly, a negative correlation between CTGF and phosphorylation status of p38 was identified in The Cancer Genome Atlas lung adenocarcinoma dataset. In the context of the clinical importance of our findings, we showed that p38 inhibitor, SB203580, reduced the metastatic potential of NSCLC secreting low levels of CTGF. Collectively, our present findings indicate that the CTGF/p38 axis is a novel therapeutic target of NSCLC metastasis, particularly NSCLC secreting low levels of CTGF.

Lung cancer is the most frequent cause of major tumor incidence and mortality worldwide.[1] When diagnosed, approximately 70% of lung cancer patients have advanced-stage disease that has already spread locally or to distant organs.[2] Non-small-cell lung cancer (NSCLC) comprises approximately 80% of all diagnosed lung cancer, and lung adenocarcinoma is the most common subtype of NSCLC. The median survival of metastatic NSCLC is still <9 months, even when treated with conventional chemotherapy or molecular-targeted therapy;[3] therefore, additional targets and novel therapeutic strategies are clearly needed for NSCLC metastasis.

Connective tissue growth factor (CTGF, also known as CCN2) is a secreted matricellular protein that belongs to a member of the cysteine-rich angiogenic inducer 61/CTGF/nephroblastoma overexpressed family of growth factors.[4] Connective tissue growth factor modulates intracellular signal pathways by associating with various cell surface molecules or extracellular ligands and is widely involved in diverse cellular processes including differentiation, proliferation, adhesion, and cell motility.[5] The physiological role of CTGF in lung tissue development and fibrosis has been well studied; it is known that CTGF mediates transforming growth factor-β signaling and promotes fibrosis in various tissues.[6,7] In addition, CTGF has been known as a tumor suppressor in ovarian and lung cancer[8,9] and the decrease of CTGF expression is associated with distant tumor metastasis incidence and poorer patient prognosis.[10,11] However, the precise molecular mechanism or the downstream signal of CTGF that suppresses tumor invasion and metastasis has not been elucidated.

In this study, we show that secreted CTGF prevents tumor invasion by suppressing the p38 pathway in NSCLC cell lines using recombinant CTGF or siRNA against CTGF. Consistent with these *in vitro* findings, *CTGF* expression is negatively correlated with the phosphorylation status of p38 in lung adenocarcinoma patients, the most major subset of NSCLC. Importantly, we report that pharmacological inhibition of p38 effectively suppresses invasion and metastasis in NSCLC secreting low levels of CTGF. In summary, we propose that the CTGF/p38 axis could be a novel therapeutic target of NSCLC, particularly those secreting low levels of CTGF.

Materials and Methods

Reagents and plasmid. Recombinant human full-length CTGF (rCTGF) was purchased from Cell Sciences (Carlsbad, CA, USA). A p38 inhibitor (SB203580) was purchased from Merck Millipore (Billerica, MA, USA). pFLAG-p38α vector was constructed as described previously.[12]

Cell culture. PC-9 was a kind gift from Dr. Katsuyuki Kiura (Okayama University, Okayama, Japan). PC-14, A549, and

PC-9 cells were cultured in RPMI-1640 medium (Nissui Seiyaku, Tokyo, Japan) with 10% FBS (Nichirei Biosciences, Tokyo, Japan), 2 mM L-glutamine (Life Technologies, Gaithersburg, MD, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin in 5% CO_2 at 37°C. A549/Luc2 cells were established as described previously.[13] PC-14/Luc2 cells were established using a similar protocol. Briefly, parental A549 or PC-14 cells were transfected with pGL4.50/Luc2 (Promega, Madison, WI, USA) and cloned by selection with 200 μg/mL hygromycin B. PC-14 and A549/Luc2 cells were treated with 100 ng/mL rCTGF for 48 h in each experiment.

For the knockdown experiment, 25 nM siCTGF #08 (S3708; Thermo Fisher Scientific, Rockford, IL, USA), siCTGF #09 (S3709; Thermo Fisher Scientific), siMAPK14 (Stealth RNA interference; Invitrogen), or siControl (Silencer Select negative control#1 siRNA; Ambion) were transfected by Lipofectamine RNAiMAX (Life Technologies) and the transfected cells were subjected to a Matrigel invasion assay or Western blotting after 72 h.

For transient transfection, pFLAG-p38α or vector control plasmids with pEGFP-C1 (Clontech, Palo Alto, CA, USA) at 5:1 ratio were cotransfected into PC-14 cells using Lipofectamine 2000 reagent (Life Technologies). Five hours after transfection, culture medium was changed to fresh complete medium (RPMI-1640 containing 10% FBS) without or with 100 ng/mL rCTGF. The transiently transfected cells were continuously cultured for 48 h and then subjected to Matrigel invasion assay or Western blotting.

Immunoassay. To assess the amount of CTGF secreted from lung cancer cells, 1×10^5 PC-9, A549, and PC-14 cells were seeded into 24-well plates and cultured for 24 h in complete medium (RPMI-1640 containing 10% FBS). The complete medium was then changed to serum-free RPMI-1640 containing 0.25% BSA and 100 μg/mL heparin, and cells were continued to culture for an additional 72 h. Finally, cell-free culture supernatants were harvested and subjected to ELISA for CTGF expression (PeproTech, Rocky Hill, NJ, USA).

***In vivo* invasion assay by i.v. inoculation.** Female 5- or 6-week-old C.B-17/lcrHsd-*Prkdc*^{scid} mice were purchased from Japan SLC (Hamamatsu, Japan). All experiments were approved and carried out according to the guidelines of the Care and Use of Laboratory Animals of University of Toyama (Toyama, Japan). A549/Luc2 or PC-14/Luc2 cells were precultured in the absence or presence of 100 ng/mL rCTGF or 10 μM SB203580 for 48 h and were inoculated i.v. (A549/Luc2, 2×10^6 cells/200 μL PBS/mouse; PC-14/Luc2, 1×10^6 cells/200 μL PBS/mouse) into mice and the lungs were removed 24 h after the tumor inoculation. Mice were i.p. injected with 200 μL luciferin (10 mg/mL VivoGlo; Promega) 20 min prior to bioluminescent assay using an *in vivo* imaging system (IVIS Lumina II; Caliper Life Sciences, Hopkinton, MA, USA). Outliers were statistically determined by the Smirnov–Grubbs test and excluded the experimental analysis. The data are presented as a box plot.

Matrigel invasion assay. Invasive potential through reconstituted basement membrane (Matrigel) was assayed as described previously.[13] Briefly, in a Transwell Boyden chamber, a polycarbonate filter with 8-μm pore size (Nucleopore Track-Etch Membrane; Whatman, Clifton, NJ, USA) was precoated with 1.25 μg fibronectin or laminin (Iwaki Glass Co. Ltd., Tokyo, Japan) on the lower surface and with 5 μg Matrigel on the upper surface of the filters. Cells pretreated with rCTGF or siCTGF (3×10^4 cells/100 μL/well) were added to the upper compartment, and incubated at 37°C for 6.5 h. The filters were fixed and stained with H&E. For detection of EGFP$^+$-invaded cells, filters were fixed with 4% paraformaldehyde and mounted by VECTASHIELD with DAPI (Vector Laboratories, Burlingame, CA, USA). The invaded cells were counted manually under a microscope at ×100 magnification or a fluorescence microscope at ×50 magnification.

Western blot analysis. Whole cell lysates were prepared as described previously.[13] Primary antibodies used were phosphorylated (p-)p38 (Thr180/Tyr182), heat shock protein (p-HSP)27 (Ser78), HSP27, p-ERK1/2 (Thr202/Tyr204), p-JNK1/2/3 (Thr183/Tyr185), protein kinase B (p-AKT) (Ser473), p-Src (Tyr416), focal adhesion kinase (p-FAK) (Tyr397), FAK, p-Smad2 (Ser465/467), Smad2/3, p-p65 (Ser536), and phospholipase Cγ1 (p-PLCγ1) (Tyr783) (Cell Signaling Technology, Beverly, MA, USA), antibodies against α-tubulin, FLAG (Sigma-Aldrich, St. Louis, MO, USA), and antibodies against p38α, ERK1, JNK1, AKT1, p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibody against human CTGF was purchased from PeproTech. Appropriate HRP-conjugated antibodies were used as secondary antibodies.

WST-8 assay. PC-14 cells were cultured in the presence or absence of 10 μM SB203580 for 48 h, and then subjected to WST-8 assay using a cell counting kit (Dojindo, Kumamoto, Japan).

Correlation analysis in TCGA clinical samples. For the correlation analysis using The Cancer Genome Atlas (TCGA) datasets, the following types of the "level3" processed and normalized dataset were downloaded from the TCGA website (https://tcga-data.nci.nih.gov/tcga/): Illumina HiSeq RNA-Seq v2 exon expression data (RPKM normalized) and reverse phase protein array (RPPA) proteomic data of lung adenocarcinoma. Because TCGA analysis of lung adenocarcinoma samples had not been completed at the time of submission of this manuscript, the RPPA dataset includes only the proteomics data for 181 out of 578 lung adenocarcinoma patients. Thus, the CTGF mRNA expression and p-p38 (pT180/pY182) protein expression status of the corresponding 181 lung adenocarcinoma samples were extracted from these datasets and used for correlation and survival analysis on March 10, 2015. The expression in each lung adenocarcinoma patient was plotted and statistically examined by R software (R Foundation for Statistical Computing, Vienna, Austria).

Statistical analysis. Statistical significance was calculated using R software. More than three means were composed using one-way ANOVA with Bonferroni correction, and two means were composed using unpaired Student's *t*-test or Mann–Whitney *U*-test. For the correlation study in clinical samples, Spearman's order correlation coefficient was used. *P*-values <0.05 were considered statistically significant.

Results

Connective tissue growth factor secretion associated with metastatic and invasive potential of NSCLC. We first determined the secretion of CTGF in human NSCLC cell lines PC-14, A549, and PC-9. As shown in Figure 1(a), we observed the various levels of CTGF secretion in those three cell lines with inverse correlations in their *in vitro* invasiveness induced by fibronectin. To further investigate the significance of CTGF in NSCLC invasiveness, we supplemented rCTGF to the culture of PC-14 cells, which secreted low levels of CTGF and were therefore highly invasive. As shown in Figure 1(b), we observed a significant suppression in the invasion of PC-14 cells without any growth suppression (data not shown).

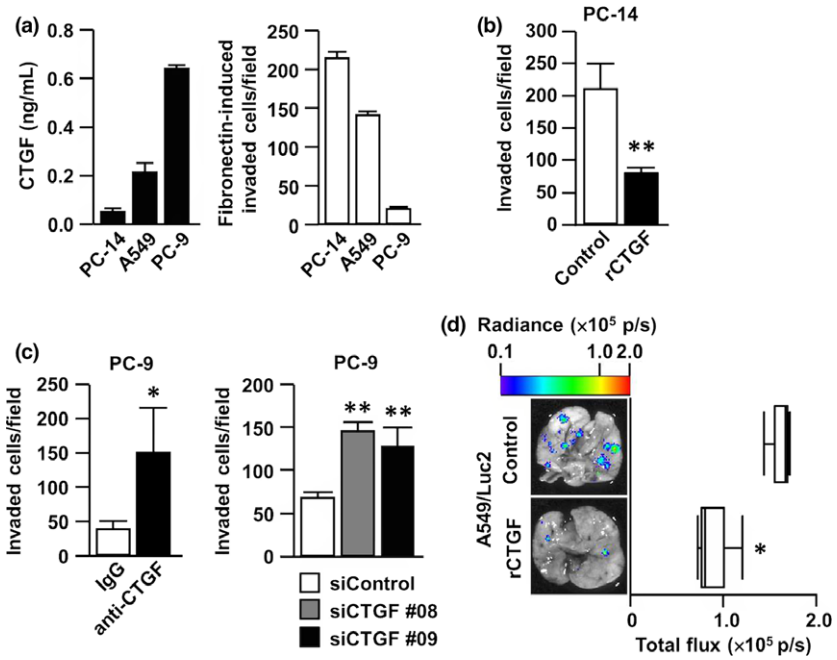

Fig. 1. Connective tissue growth factor (CTGF) suppresses tumor invasion potential in non-small-cell lung carcinoma (NSCLC) *in vitro* and *in vivo*. (a) The conditioned mediums from three NSCLC cell lines were subjected to ELISA for detecting secreted CTGF (left panel). Three NSCLC cell lines were subjected to Matrigel invasion assay using human fibronectin as an attractant (right panel). Data represents the mean ± SD of triplicate experiments. (b) PC-14 cells were pretreated with 100 ng/mL recombinant CTGF (rCTGF) for 48 h and subjected to Matrigel invasion assay using fibronectin as an attractant. (c) PC-9 cells were subjected to Matrigel invasion assay using laminin as an attractant after treatment with 10 μg/mL anti-CTGF neutralizing antibody for 48 h (left panel) or siCTGF for 72 h (right panel). Data represent the mean ± SD of triplicate experiments. $^*P < 0.05$, $^{**}P < 0.01$ *versus* Control group by two-tailed Student's *t*-test. (d) A549 cells overexpressing the *Luc2* gene (A549/Luc2) were subjected to *in vivo* experimental metastasis following 48 h of culture in the absence or presence of rCTGF. Mice were sacrificed 24 h after tumor inoculation and lungs were subjected to bioluminescent imaging to determine total flux (photon/s [p/s]) for lung metastasis quantification. The representative *ex vivo* images are shown. Data are shown as a box plot ($n = 4$). $^*P < 0.05$ *versus* Control by two-tailed Mann–Whitney *U*-test.

Conversely, the increased invasiveness was detected by using neutralizing antibody or siRNA against CTGF (anti-CTGF or siCTGF) in PC-9 cells (Fig. 1c), which secreted the highest levels of CTGF and showed the lowest invasiveness among three NSCLC cell lines (Fig. 1a). Importantly, the pretreatment with rCTGF significantly suppressed *in vivo* metastatic spread of A549/Luc2 cells in the experimental lung metastasis model (Fig. 1d). Collectively, these results indicate the importance of secreted CTGF in the metastatic behavior of NSCLC cells.

P38 pathway is a key downstream signal of CTGF to regulate metastatic potential of NSCLC. Considering the controversial effect of CTGF in cancer cell metastatic behavior in different tissues,[8–11,14–17] we decided to identify the drug-able molecules downstream of CTGF in NSCLC cells, which is potentially involved in suppressing NSCLC metastasis. By focusing on the 10 protein kinases that are known to be regulated by CTGF (p38, HSP27, ERK1/2, JNK, protein kinase B [AKT], focal adhesion kinase [FAK], Src, Smad2, p65, and PLCγ1),[18] we found that p38 and HSP27 phosphorylation were significantly suppressed by rCTGF treatment in PC-14 cells (Fig. 2a). In contrast, siRNA against CTGF enhanced the phosphorylation of p38 and HSP27 in PC-9 cells (Fig. 2b). We also detected the suppression of p-ERK1/2 and induction of p-JNK1/2/3 in PC-9 cells; these phosphorylations could not be seen in PC-14 cells, suggesting differences in each cell line. Given HSP27 is known as a terminal substrate of the p38 cascade,[19] we next checked the correlation between CTGF mRNA and p-p38 (pT180/pY182) expression levels in 181 TCGA lung adenocarcinoma samples using exome sequences and the RPPA dataset. Consistent with our Western blotting data, CTGF mRNA expression was negatively correlated with p-p38 expression (Fig. 2c), suggesting the molecular link between CTGF secretion and the p38 pathway in NSCLC cells.

To directly determine whether the activation status of p38 contributes to CTGF-mediated regulation of NSCLC metastasis, we established a cell line overexpressing p38 using PC-14 cells, which secrete low levels of CTGF. As shown in Figure 3(a), the reduced invasiveness by rCTGF treatment was consistently observed in control transfected cells (MOCK). While significant upregulation of invasiveness of PC-14 cells was seen by overexpressing p38a (FLAG-p38 in Fig. 3a), the inhibitory effect of rCTGF was largely diminished in FLAG-p38 PC-14 cells (Fig. 3a). In addition, N-cadherin suppression was also diminished by overexpression of p38. Conversely, the induction of invasiveness by transducing siCTGF was suppressed by knocking down *MAPK14*, the official gene name for p38α, in PC-9 cells (Fig. 3b). In concert with the invasiveness, we could detect the induction of N-cadherin expression by siCTGF, which was diminished by siMAPK14. Consistently, a selective p38 kinase inhibitor, SB203580, suppressed siCTGF-induced invasive potential in A549 cells (Fig. S1). These results clearly indicate that CTGF suppresses the invasive and metastatic potential of NSCLC cells through the inhibition of the p38 pathway.

P38 is a potential therapeutic target for NSCLC metastasis secreting low levels of CTGF. To explore the possibility of p38 as a new therapeutic target for metastatic inhibition of NSCLC with low secretion of CTGF, we examined the effect of selective p38 inhibitor, SB203580,[20–22] on invasion, proliferation,

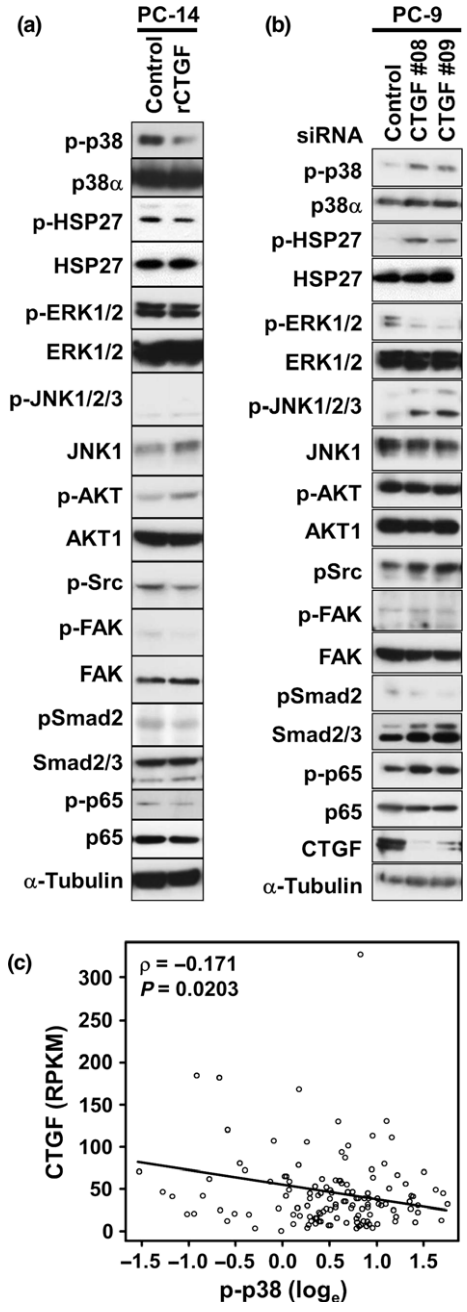

Fig. 2. Connective tissue growth factor (CTGF) prevents the p38 MAPK pathway in non-small-cell lung carcinoma cells. (a, b) Western blotting for the expression of various phosphorylated (p-) protein kinases in PC-14 cells following treatment with 100 ng/mL recombinant CTGF (rCTGF) for 48 h (a) or in PC-9 cells following siCTGF treatment for 72 h (b). (c) Correlation between CTGF mRNA and p-p38 (pT180/pY182) protein expression in 181 lung adenocarcinoma cases from The Cancer Genome Atlas using Spearman's order correlation coefficient ($\rho = -0.171$, $P = 0.0203$). *y*-Axis shows the normalized CTGF mRNA expression (read per kilobase/million [RPKM]) in RNA-Seq data. *x*-Axis defines the normalized and natural log-transformed expression of p-p38 in reverse phase protein array data. Each dot indicates the expression level in an individual patient. AKT, protein kinase B; FAK, focal adhesion kinase; HSP, heat shock protein.

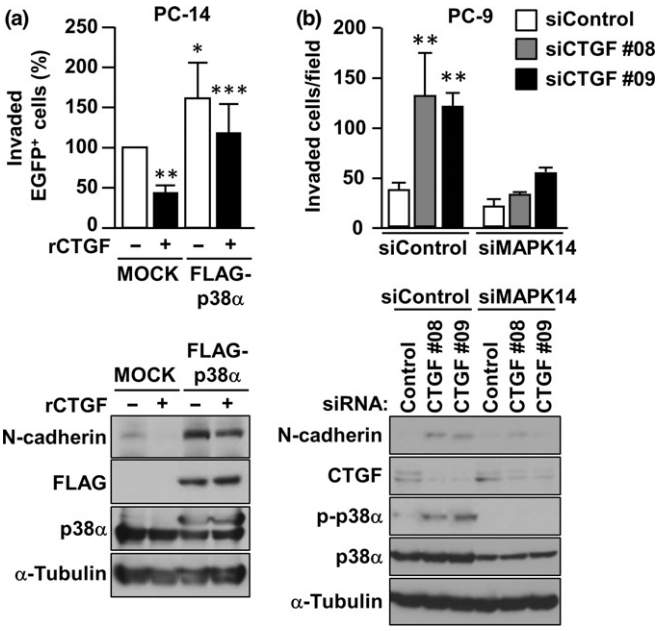

Fig. 3. Connective tissue growth factor (CTGF) suppresses tumor invasion potential through the inhibition of p38 in non-small-cell lung carcinoma cells. (a) PC-14 cells were cotransfected with p38α expression vector and EGFP expression vector. After 5 h, cells were treated with 100 ng/mL recombinant CTGF (rCTGF) for 48 h and subjected to Matrigel invasion assay (upper panel) or Western blotting (lower panel). Invaded EGFP$^+$ cells were counted manually under a fluorescence microscope at 50× magnification. Data are the means ± SD of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ *versus* rCTGF(−) in the MOCK group. $^{***}P < 0.01$ *versus* rCTGF(+) in the MOCK group by two-way ANOVA followed by Bonferroni's *post-hoc* test. (b) PC-9 cells were transfected with the indicated siRNAs for 72 h and subjected to Matrigel invasion assay (upper panel) or Western blotting (lower panel). Data are the means ± SD of three independent experiments. $^{**}P < 0.01$ *versus* siControl in the siControl group by two-way ANOVA followed by Bonferroni's *post-hoc* test.

and *in vivo* metastasis in PC-14 cells. While the pharmacological inhibition of p38 by SB203580 or siMAPK14 effectively suppressed invasiveness (Figs 4a,S2) and the activation of its downstream target, HSP27 (Fig. 4c), in PC-14 cells, there was a small but significant reduction in their cell viability following treatment with SB203580 (Fig. 4b). However, the anti-invasive and antiproliferative effect of SB203580 was weaker in A549 cells, which secrete higher levels of CTGF, than in PC-14 cells (Fig. S3). Finally, we evaluated the effect of p38 inhibitor on lung metastasis of PC-14/Luc2 cells (Fig. 4d). Consistent with our *in vitro* findings, pretreatment with SB203580 significantly compromised the metastatic potential of PC-14/Luc2 cells. Taken together, our results suggest that p38 can be a therapeutic target for inhibition of metastasis in NSCLC, particularly those secreting low levels of CTGF.

Discussion

In the present study, we revealed the molecular link between CTGF secretion and the p38 pathway in the invasive and metastatic potential of NSCLCs. Importantly, a negative correlation between CTGF and phosphorylation status of p38 was identified in TCGA lung adenocarcinoma dataset. In terms of the clinical importance of our findings, we showed that p38 inhibitor, SB203580, reduced the metastatic potential of NSCLC cells, particularly those secreting low levels of CTGF.

Although CTGF has been known to be related to metastasis in lung cancer,[10,11] the precise mechanism by which CTGF regulates lung cancer metastasis has not been elucidated. In addition to our findings regarding the molecular link between

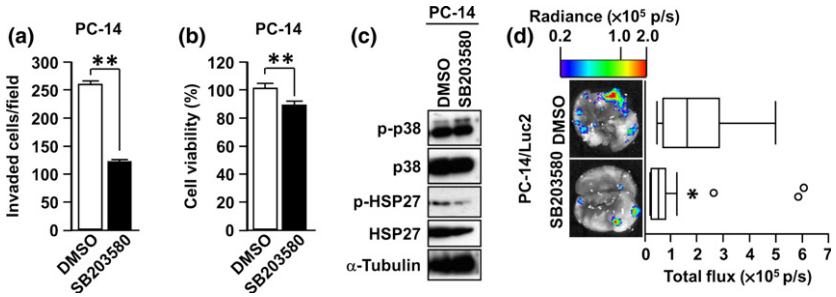

Fig. 4. P38 could be a therapeutic target for tumor metastasis in non-small-cell lung carcinomas secreting low levels of connective tissue growth factor (CTGF). (a–c) PC-14 cells were treated with 10 μM SB203580 (black column) or DMSO (white column) for 48 h and subjected to Matrigel invasion assay (a), WST-8 assay (b), and Western blot analysis (c). $^{**}P < 0.01$ *versus* DMSO group by two-tailed Student's *t*-test. (d) PC-14 cells overexpressing the *Luc2* gene (PC-14/Luc2) were treated with 10 μM SB203580 or DMSO for 48 h and continuously subjected to *in vivo* experimental metastasis assay. Lungs were subjected to bioluminescent imaging for lung metastasis quantification. Representative *ex vivo* images are shown. Data are represented as a box plot ($n = 12$). Outliers were statistically determined by Smirnov–Grubbs test and shown as open circles. $^{*}P < 0.05$ *versus* DMSO group by two-tailed Mann–Whitney *U*-test.

CTGF and the p38 pathway, we also noted the suppression of N-cadherin expression by rCTGF treatment and siCTGF induced *COX-2* mRNA (Figs S4,S5). Considering *COX-2* is known as a target gene of the p38 pathway[23] and an upstream gene of N-cadherin, and N-cadherin is also known as a mesenchymal marker, the induction of mesenchymal–epithelial transition might be a potential mechanism regulating the invasion and metastasis of NSCLC by CTGF. Consistent with our hypothesis and previous reports,[10] we observed mesenchymal–epithelial transition-like morphological and molecular changes by CTGF treatment in PC-14 cells, but epithelial–mesenchymal transition-like changes by CTGF silencing in A549 and PC-9 cells (Figs S5,S6). Alternatively, CTGF is also known to induce anoikis through the inhibition of the ERK pathway,[10] which is also key for cell survival during metastatic spread of cancer cells.[24] Collectively, we speculate that CTGF suppresses NSCLC metastasis cooperatively by inhibiting invasiveness through the p38/COX-2/N-cadherin pathway and by inducing anoikis through inhibition of the ERK pathway. However, further study is clearly required.

In contrast to NSCLCs, CTGF has been known to promote tumor development in breast,[15] pancreatic,[14] gastric cancer,[17] and glioma,[16] therefore the direct targeting of CTGF may not be applicable for treating cancer metastasis due to those potential risks. Instead, we identified p38 as a critical molecule downstream of CTGF in NSCLCs that could be a drug-able target to control their metastasis. Even though the efficacy of targeting the p38 pathway can be preferentially expected in NSCLCs secreting low levels of CTGF, our results provide a new therapeutic opportunity for NSCLC metastasis by compensating a dysregulation of the CTGF/p38 axis. Furthermore, we found longer relapse-free survival rates in $CTGF^{high}$/$p\text{-}p38^{low}$ patients compared to $CTGF^{low}$/$p\text{-}p38^{high}$ patients (Fig. S7; hazard ratio = 2.454, $P = 0.0371$) by analyzing the same TCGA dataset used in Figure 2(c). Considering the homologous deletion of the 6q23 locus, which contains the *CTGF* gene, has been reported in some lung cancer patients,[25] and the loss of CTGF expression has been known to account for selective activation of p38 in lung cancer specimens,[26] we also propose the utility of the CTGF/p38 axis as a potential biomarker for metastasis risk of lung cancer patients.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Effect of p38 inhibitor, SB203580, on siCTGF-induced invasion in A549 non-small-cell lung carcinoma cells.

Fig. S2. Effect of p38 knockdown on invasive potential of PC-14 non-small-cell lung carcinoma cells.

Fig. S3. Effect of p38 inhibitor, SB203580, on invasive and proliferative potential of A549 non-small-cell lung carcinoma cells.

Fig. S4. Expression of COX-2 mRNA following connective tissue growth factor (CTGF) knockdown.

Fig. S5. Expression of E- and N-cadherin following recombinant connective tissue growth factor (rCTGF) treatment or CTGF knockdown.

Fig. S6. Morphological changes in non-small-cell lung carcinomas treated with recombinant connective tissue growth factor (rCTGF) or CTGF knockdown.

Fig. S7. Kaplan–Meier curve of connective tissue growth factor $(CTGF)^{high}$/p-$p38^{low}$ and $CTGF^{low}$/p-$p38^{high}$ patients using The Cancer Genome Atlas dataset of 181 non-small-cell lung carcinoma cases.