Inhibition of tumor invasion and metastasis by targeting TGF-β-Smad-MMP2 pathway with Asiatic acid and Naringenin

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Transforming growth factor β (TGF-β) has been shown to promote tumor invasion and metastasis by activating the matrix metalloproteinases (MMPs); however, signaling mechanisms remain controversial and therapies targeting MMPs are still suboptimal. In the present study, we found that combined therapy with Asiatic acid (AA), a Smad7 agonist, and Naringenin (NG), a Smad3 inhibitor, effectively retrieved the balance between Smad3 and Smad7 signaling in the TGF-β-rich tumor microenvironment and thus significantly suppressed tumor invasion and metastasis in mouse models of melanoma and lung carcinoma. Mechanistically, we unraveled that Smad3 acted as a transcriptional activator of MMP2 and as a transcriptional suppressor of tissue inhibitors of metalloproteinase-2 (TIMP2) via binding to 5’ UTR of MMP2 and 3’ UTR of TIMP2, respectively. Treatment with NG inhibited Smad3-mediated MMP2 transcription while increasing TIMP, whereas treatment with AA enhanced Smad7 to suppress TGF-β/Smad3 signaling, as well as the activation of MMP2 by targeting the nuclear factor-κB (NF-κB)-membrane-type-1 MMP (MT1-MMP) axis. Therefore, the combination of AA and NG additively suppressed invasion and metastasis of melanoma and lung carcinoma by targeting TGF-β/Smad-dependent MMP2 transcription, post-translational activation, and function.

INTRODUCTION

Invasion and metastasis are the major causes of death in both melanoma and lung carcinoma at stage IV.2,2 Although it has been regarded as a negative regulator for tumor cell proliferation, transforming growth factor β (TGF-β) has been long considered as a promoter for tumor invasion and metastasis.3,5 Melanoma is notorious for its predilection for metastasis even at an early stage of progression.6 Although melanoma is resistant to TGF-B1-mediated growth inhibition, the development of melanoma osteolytic bone metastases was significantly reduced by blocking TGF-β receptor I kinase (TBRI).7,8 It has also been reported that high-level TGF-β is closely correlated with enhanced migration capability of non-small cell lung cancer (NSCLC) and the incidence of lymph node metastasis.9,10 TGF-β-induced tumor invasion and metastasis are closely related to the proteolytic activity of matrix metalloproteinases (MMPs).11 MMPs are a zinc-dependent endopeptidase implicated in basement membrane degradation, angiogenesis, and cell migration, thereby contributing to tumor invasion and metastasis.12 Among the family of MMPs, MMP2 plays a crucial role for the remodeling of basement membrane via degrading collagen I, IV, V, plasminogen, and laminin-5, hence promoting angiogenesis and paving a way for tumor cell migration as seen in melanoma and lung carcinoma.13-16 MMP2 also induces angiogenesis via mediating the expression of important angiogenic factors like vascular endothelial growth factor (VEGF) and MMP9.17,18 In addition, the final maturation of invadopodia, which are actin-based protrusions in highly invasive and metastatic tumor cells, requires the presence of MMP2 as well.19 Previous research has found that TGF-B1-driven melanoma metastasis is correlated with the expression, activation, and tissue inhibitors of metalloproteinase-2 (TIMP2)-dependent inhibition of MMP2.20 Moreover, MMP2 overexpression is correlated with lymph node metastasis and unfavorable outcome in NSCLC patients.21,22

Many clinical trials have been performed with several generations of synthetic inhibitors of MMPs (MMPIs), however, all trials failed largely due to the low specificity and severe musculoskeletal side effects of MMPIs.23,24 Thereby, TGF-β/Smad signaling could be potential therapeutic targets to suppress MMP2-dependent tumor invasion and metastasis. Previously we found that the combination of two compounds isolated from herbal medicine, Asiatic acid (AA), a triterpene from Centella asiatica that functions as a Smad7 enhancer, and Naringenin (NG), a type of flavonoid mainly present in fruits as a

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Smad3 inhibitor, exert potent anti-cancer effects by rebalancing Smad3/Smad7 signaling and subsequently suppresses cancer progression by rejuvenating natural killer (NK) cell-mediated immune surveillance. In the present study, we hypothesized that TGF-β promotes tumor invasion and metastasis via Smad3-dependent MMP2-mediated pathways and this may also be the mechanism whereby combined therapy of AA and NG effectively suppresses melanoma and lung carcinoma invasion and metastasis.

RESULTS
Combination of AA and NG can effectively suppress melanoma and lung carcinoma invasion and metastasis in vivo and in vitro
We first investigated the anti-invasive and metastatic effects of AA and NG on syngeneic mouse models of melanoma and lung carcinoma in C57BL/6 mice with intact tumor microenvironment. As determined by a dose-dependent study as described previously, AA at a dose of 10 mg/kg/day and NG at a dose of 50 mg/kg/day produce effective anti-cancer effects in these syngeneic mouse models. In the present study, mice bearing B16F10 melanoma and Lewis Lung Cancer (LLC) were treated with either AA or NG at their optimized dose or the combination of these two drugs daily intraperitoneally (i.p.) for 4 weeks. As shown in Figures 1A and 1G, subcutaneous tumor invasions were frequently found in mice without treatment, usually accompanied by noticeable angiogenesis. Meanwhile, metastatic nodules of melanoma and lung carcinoma in liver, lung, and intestine were commonly seen in mice without treatment (Figures 1C–1E). In contrast, either AA or NG treatment showed a significant inhibition on tumor invasion and metastasis, and these inhibitory effects were

![Figure 1](image-url)
A

|       | Ctrl | TGF-β1 | AA+TGF-β1 | NG+TGF-β1 | CB+TGF-β1 |
|-------|------|--------|-----------|-----------|-----------|
| 0h    |      |        |           |           |           |
| 6h    |      |        |           |           |           |
| 12h   |      |        |           |           |           |
| 24h   |      |        |           |           |           |

B

|       | Ctrl | TGF-β1 | AA+TGF-β1 | NG+TGF-β1 | CB+TGF-β1 |
|-------|------|--------|-----------|-----------|-----------|

C

|       | Ctrl | TGF-β1 | AA+TGF-β1 | NG+TGF-β1 | CB+TGF-β1 |
|-------|------|--------|-----------|-----------|-----------|

D

Wound healing assay

E

Tranwell assay

F

Colony formation assay

(legend on next page)
further enhanced in mice receiving a combined treatment, where both melanoma and lung carcinoma developed in an encapsulated growth pattern subcutaneously (Figures 1B and 1F). These therapeutic effects on melanoma and lung carcinoma invasion and metastasis were quantitatively analyzed in Figures 1H and 1I.

We next examined the inhibitory effects of AA and NG on TGF-β1-induced LLC cell migration and invasion in vitro by wound healing, transwell, and colony-formation assay. Results of wound-healing assay in Figures 2A and 2D demonstrated that individual AA and NG inhibited TGF-β1-induced migration of LLC cells by 50% and 68%, respectively, while combined treatment further reduced the migration of LLC cells by 80%. Similar results were also found in a transwell assay in which either AA or NG alone was capable of inhibiting TGF-β1-induced LLC migration, and this inhibition was further strengthened by the combined treatment (Figures 2B and 2E). Moreover, colony-formation assay also revealed that treatment with AA or NG significantly suppressed TGF-β1-induced clonogenic formation of LLC cells, which was additively enhanced by the combination of AA and NG (Figures 1C and 1F). These data suggest that AA and NG are promising inhibitors for LLC migration, invasion, and metastasis. However, MTT assay implied that combined therapy induced a slight inhibition on LLC cell viability and proliferation, which may partially contribute to the decrease in LLC cell migration, invasion, and metastasis (Figure S1).

**Inhibition of tumor metastasis by combination of AA and NG is associated with the suppression of MMP2**

Since TGF-β promotes tumor invasion and metastasis via MMP-dependent mechanisms, we then examined the expression of MMPs in LLC with or without treatment of AA and NG. As shown in Figure 3A, TGF-β/Smad signaling was altered with highly activated Smad3 but reduced Smad7 signaling, which was associated with upregulated expression of MMP2, MMP9, and MMP13 in LLC lung carcinoma. Treatment with AA upregulated Smad7, whereas NG inhibited activation of Smad3, both resulting in a suppression of MMP9 without significantly altering MMP2 and MMP13 expression. In contrast, combined treatment with AA and NG additively inactivated Smad3 while it increased Smad7 expression, meanwhile significantly blocking MMP2, MMP9, and MMP13 expressions (Figure 3A). At transcriptional levels, treatment with NG partially inhibited the transcription of MMP2, MMP3, and MMP9 but not MMP13, whereas treatment with AA slightly reduced MMP9 and MMP13 expression without affecting MMP2 and MMP3 with no statistical significance (Figures 3B–3E). In contrast, LLC-bearing mice treated with the combination of AA and NG resulted in a signaling inhibition of all MMPs examined (Figures 3B–3E). Notwithstanding, a more potent suppression on MMP2 was achieved by the combined therapy compared to AA or NG therapies alone (Figures 3A and 3B), which was correlated with better inhibitory effects on invasion and metastasis in mouse models (Figure 1) and in vitro studies (Figure 2). Thus, MMP2 may be an essential mediator for cancer invasion and metastasis and, more importantly, as a key therapeutic mechanism in response to combined therapy with AA and NG.

To confirm the role of MMP2 in invasion and metastasis of melanoma and lung carcinoma, we examined the expression of MMP2 in both metastatic niches and the primary tumor sites. As shown in Figures 4A and 4B, MMP2 was highly expressed in liver metastatic niches of LLC. We also detected high expressions of MMP2 at the edge of invasive LLC and B16F10 melanoma (Figures 4C and 4D). Treatment with either AA or NG alone caused a 30%–60% decrease in MMP2-producing cells in the primary tumor sites in both of the two mouse models, which was further reduced by 70% in B16F10 melanoma (Figures 4D and 4F) and 90% in LLC lung carcinoma (Figures 4C and 4E) after receiving the combined therapy. This marked decrease in MMP2 expression induced by combined therapy is closely related to the enhancement of Smad7 and suppression of p-Smad3 levels in LLC lung carcinoma (Figure S2).

Taken together, these data indicated that combined therapy of AA and NG, through rebalancing Smad3/Smad7 signaling, suppressed TGF-β1-mediated MMP2 expression and inhibited lung carcinoma and melanoma invasion and metastasis.

**Combination of AA and NG treatment inhibits TGF-β1-mediated MMP2 transcription, activation, and function via rebalancing Smad3/Smad7 signaling**

To further explore the underlying mechanisms through which AA and NG inhibit the pro-metastatic activity of MMP2, the influence of individual and combined therapy of AA and NG on MMP2 transcription, activation, and function were determined on TGF-β1-stimulated LLC cells in vitro.

First, we noticed that TGF-β1 induced an increase of pro-MMP2 (72 kDa) and active MMP2 (63 kDa) in a dose-dependent manner, peaking at the dosage of 5 ng/mL (Figure 5A). Therefore, 5 ng/mL TGF-β1 was selected as an optimal dosage for induction of MMP2 in subsequent in vitro experiments. Both the levels of pro-MMP2 and active MMP2 were positively correlated with the expressions of p-ALK5 and p-Smad3 (Figure 5A), suggesting an involvement of canonical TGF-β1/Smad3 signaling in the transcriptional and translational regulations of MMP2. Interestingly, we noticed that the increment of active MMP2 induced by TGF-β1 was much greater than that of pro-MMP2, suggesting the possibility of post-translational regulations, such as the activation of pro-MMP2 through membrane-type-1 MMP (MT1-MMP).
We then investigated the influence of AA and NG pre-treatment on MMP2 translation, activation, and function. As demonstrated in Figure 5B, the combined therapy with AA and NG achieved better rebalance of Smad3/Smad7 signaling by reducing phosphorylation of ALK5 and Smad3 while upregulating Smad7 as compared with individual therapies. This was associated with inhibition of NF-κB p50 signaling, pro-MMP2, and active MMP2 expression. Additionally, results from real-time PCR also showed that TGF-β1 induced MMP2 transcription in a dose-dependent manner, which was effectively inhibited by both AA and NG (Figures 5C and 5D).

Interestingly, although both individual AA and NG suppressed the upregulation of pro-MMP2, only the pre-treatment of AA inhibited the activation of MMP2, indicating divergent post-translational regulations induced by AA and NG. Further investigation on MT1-MMP and TIMP2 validated our hypothesis that AA largely prevented the expression of MT1-MMP to limit pro-MMP2 activation, while NG enhanced expression of TIMP2 to limit the proteolytic activity of MMP2 (Figure 5B).

TGF-β1 is capable of inducing the transcription of MMP2 and TIMP2 directly via Smad signaling and promoting the activation of pro-MMP2 through crosstalk of NF-κB signaling

Although TGF-β1-mediated MMP2 expression has been reported to be transcriptionally regulated via crosstalk to ATF2/AP1, Ets1, and p38 mitogen-activated protein kinase (MAPK) signaling,27–31 the present study further elucidated that TGF-β1 directly induced MMP2 and suppressed TIMP2 expression via Smad signaling. As shown in Figure 6A, a predicted conserved Smad binding site between mouse and human was found on the 5' UTR of MMP2 with Evolutionary Conserved Regions (ECR) brower (rVista 2.0, https://rvista.dcode.org/).32,33 Then, specific primers covering the predicted Smad binding site were designed for the chromatin immunoprecipitation (ChIP) assay (Figure 6B). From the results of Figure 6C, TGF-β1 stimulation greatly enhanced the binding of p-Smad3 to the predicted Smad binding site on the 5' UTR of MMP2. A dual-luciferase assay was carried out to further validate the regulatory role of Smad3 on the MMP2 transcription. The transfection of a pcDNA3.1+Smad3 plasmid significantly increased the promoter activity, which was abrogated by the induction of point mutations of the predicted Smad binding site (Figure 6D). Taken together, we confirmed that Smad3 mediated TGF-β1-induced MMP2 expression as a transcriptional activator.

With respect to the regulation of TGF-β1 on TIMP2, a Smad binding site was also discovered on the 3' UTR of the TIMP2 gene (Figure 6E).
As shown in Figure 6F, addition of TGF-β1 induced an increase in the interaction between p-Smad3 and the predicted binding site on TIMP2, suggesting that TGF-β1 may strengthen the proteolytic function of MMP2 partially through the inhibition of TIMP2.

Regarding the regulatory mechanisms of TGF-β1/Smad signaling on MT1-MMP expression, a NF-κB p50 predicted binding site conserved between human and mouse on the intronic region was found 56 bp downstream of the first exon of the MT1-MMP gene (Figure 6G). ChIP assay revealed an enhanced binding between phosho-NF-κB p50 (p-p50) and the predicted binding site on MT1-MMP by the activation of NF-κB signaling with LPS stimulation (Figure 6H), indicating NF-κB as a transcriptional regulator of MT1-MMP.

Figure 4. MMP2 is highly expressed in tumor metastatic niches and tumor microenvironment and is largely suppressed by combined therapy with AA and NG

(A and B) H&E staining (A) and MMP2 immunostaining (B) of LLC liver metastatic niches. Scale bar, 100 μm. (C) Immunohistochemistry detecting MMP2 at the edge of LLC lung carcinoma. (D) Immunofluorescence staining for MMP2 at the edge of B16F10 melanoma. Scale bar, 200 μm. (E and F) Quantitative results of MMP2+ cells in tumor microenvironment of (E) LLC lung carcinoma and (F) B16F10 melanoma. Each bar represents the mean ± SD for groups of three to four mice. **p < 0.01, ***p < 0.001 compared to Ctrl; #p < 0.05, ##p < 0.01, ###p < 0.001 as indicated.
Figure 5. Rebalancing Smad3/Smad7 signaling with AA and NG produces better inhibitory effects on TGF-β1-induced MMP2 transcription, activation, and function

(A and B) Western blot detecting expression of (A) p-ALK5, p-Smad3, and pro- and active-MMP2 with different doses of TGF-β1 stimulation, (B) p-ALK5, p-Smad3, Smad7, NF-κB p50, pro- and active-MMP2 (M2), MT1-MMP, and TIMP2 in LLC cells with AA and NG treatment under TGF-β1 stimulation at 5 ng/mL. (C and D) Real-time PCR shows TGF-β1 induces MMP2 transcription in a dose-dependent manner, which is inhibited by AA, NG, and combined therapy (CB). Each bar represents the mean ± SD for groups of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 compared to TGF-β1; #p < 0.05, ##p < 0.01, ###p < 0.001 as indicated.
Figure 6. TGF-β1 promotes MMP2 transcription, activation, and function via transcriptional regulations of MMP2, MT1-MMP, and TIMP2, respectively

(A and B) Smad3-binding site on the 5' UTR of MMP2 predicted by ECR browser. (C) ChIP assay and (D) dual luciferase reporter assay demonstrate that Smad3 acts as a transcriptional activator for MMP2. Each bar represents the mean ± SD for groups of three independent experiments. ***p < 0.001 as indicated; ns, no significant difference.

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Given that ChIP assays indicated possible interactions of Smad3 on TIMP’s 3’ UTR and p-p50 on MT1-MMP’s non-coding regulatory region, real-time PCR was performed afterwards to confirm the transcriptional regulations of Smad3 on TIMP2 and p-p50 on MT1-MMP, respectively. As shown in Figure 6J, both AA and NG enhanced the mRNA level of TIMP2 markedly, while the combined therapy induced an additional increase. Meanwhile, both individual AA and combined therapy can effectively attenuate TGF-β1-induced upregulation of MT1-MMP at mRNA level (Figure 6J).

DISCUSSION

TGF-β-induced MMPs are potent inducers of invasion and metastasis at late stages of tumor progression. However, the regulatory mechanisms remain poorly understood and therapies directly targeting MMPs are not satisfying. In the present study, high expressions of MMP2 were observed at the edge of tumor sites and in liver metastatic niches in both B16F10 melanoma and LLC lung carcinoma-bearing mice, indicating a pivotal role MMP2 played in melanoma and lung carcinoma metastasis.

The proteolytic activity of MMP2 is under strict control to prevent destruction of normal tissue, including transcriptional and translational regulations, activation via removing pro-peptide domain by MT1-MMP, compartmentalization, and the inhibitory effect of TIMP2. Mechanistically, we identified that TGF-β1 via Smad3 induced the transcription of MMP2 while suppressing the expression of TIMP2 to promote the proteolytic activity of MMP2. In addition, TGF-β1 also downregulated Smad7, an inhibitor of NF-κB signaling, to enhance MT1-MMP-dependent pro-MMP2 activation. Thus, unbalanced TGF-β/Smad signaling with overactive Smad3 and impaired Smad7 signaling in the tumor microenvironment may promote MMP2-dependent cancer invasion and metastasis.

Most importantly, we found that the combination of AA and NG produced a better therapeutic effect on invasion and metastasis of B16F10 melanoma and LLC lung carcinoma by additively suppressing Smad3 activation while increasing Smad7 expression compared with individual therapies. This additive suppression may attribute to the synergic inhibitory effects of AA and NG on TGF-β1/Smad signaling and its crosstalk with NF-κB signaling. NG was found to suppress TGF-β1/Smad signaling both on the receptor level and the downstream R-Smads level, while AA, through enhancing Smad7, attenuated TGF-β1-induced p-Smad3 and inhibited p-ALK5 via its negative feedback mechanism, which subsequently suppressed MMP2 while promoting TIMP2. Moreover, it has been reported that the crosstalk between TGF-β1/Smad and NF-κB signaling pathway is under the regulation of Smad7, which inhibits TGF-β1-induced TAK1-mediated phosphorylation of IκB-α and eventually enhances the activation of NF-κB signaling. Thus, AA treatment attenuated TGF-β1-induced MMP2 transcription and proteolytic function by blocking TGF-β1/Smad signaling, as well as prevented MT1-MMP-dependent pro-MMP2 activation via Smad7-mediated inhibition on TGF-β1/NF-κB/MT1-MMP signaling. To summarize, the combined therapy with AA as a Smad7 enhancer, and NG as a Smad3 inhibitor, achieved a better inhibitory effect on MMP2-dependent cancer invasion and metastasis compared with individual treatments. However, it should also be pointed out that the therapeutic effects of combined therapy on tumor invasion and metastasis may also be partially attributed to the rejuvenation of immune surveillance, as well as the inhibition of cancer cell viability and proliferation.

Accumulating evidence sheds light on the transcriptional regulation of TGF-β on MMP2 expression via both canonical Smad signaling and crosstalk of p38 MAPK, ERK, and Snail/SIP1/Ets1 pathways. According to cis-element bioinformatics analysis of promoter regions, the most commonly seen transcriptional regulators for MMP2 are STAT, DR1, NF-κB, p53, AP-2, Sp1, and CREB. Because natural herb-derived compounds are known to possess a wide spectrum of pharmacological activities, hence AA, and particularly NG, may also regulate the expression of MMP2 via non-Smad pathways such as the AKT, ERK1/2, p53, MAPK/p38, and JNK signaling pathways. We previously reported that TGF-β upregulates MMP2 in a Smad2-dependent manner in renal fibrosis, and knockdown of either Smad3 or Smad4 also largely alleviates TGF-β-induced MMP2 expression. Nevertheless, the underlying mechanisms governing the regulation remain unclear. In the present study, by using ChIP and reporter assay, we further elucidated that Smad3 functioned as a transcriptional regulator rather than as a transcriptional coactivator of Smad2 or other transcription factors to induce MMP2 transcription under TGF-β1 stimulation.

Besides MMP2, combined therapy of AA and NG also suppressed the expression of MMP3, MMP9, MMP13, and MT1-MMP. MMP3, MMP13, and MT1-MMP are potent regulators of electron cryomicroscopy (ECM) remodeling, thereby facilitating the spreading of tumor cells from primary sites, while MMP2 and MMP9 are capable of inducing angiogenesis and the formation of pre-metastasis niches, thus further promoting tumor metastasis. The increase in MT1-MMP and active form of MMP2 also contribute to the activation of pro-MMP9, which can be abrogated by TIMP1 or TIMP2. Accordingly, the combined therapy of AA and NG diminished ECM degradation mediated by MMP2 and MMP9 via suppressing MT1-MMP and enhancing TIMPs.

On the other hand, MMPs also amplify TGF-β signaling as MMP2, MMP9, and MT1-MMP can cleave the latent TGF-β-binding protein 1 (LTBP1) to solubilize ECM-bound TGF-β and MMP2, MMP9, and MMP13 can cleave the latency-associated peptide (LAP) to activate the latent TGF-β. Hence, effective inhibition of the
enzymatic functions of MMP2, MMP9, MMP13, and MT1-MMP with combination of AA and NG largely abrogated the malignant feedback amplification loop and thus attenuated TGF-β-induced tumor invasion and metastasis.

In summary, this study uncovered the mechanisms through which TGF-β1/Smad signaling regulates the transcription, post-translation, and function of MMP2. We identified Smad3 as a transcriptional regulator for MMP2 and TIMP2, and Smad7 as an inhibitor of both TGF-β/Smad3 and NF-κB signaling to block Smad3-dependent MMP2 transcription and NF-κB-induced MT1-MMP-mediated proteolytic activation of pro-MMP2. The combined therapy of AA and NG exerted potent inhibition on TGF-β1-induced MMP2-dependent melanoma and lung carcinoma invasion and metastasis through retrieving the balance of Smad3/Smad7 signaling. These results implicate a great clinical potential of the combination of AA and NG as a promising therapy for invasive and metastatic cancers such as melanoma and lung carcinoma.

MATERIALS AND METHODS

Cell culture in vitro
B16F10 cells (CRL-6475, ATCC) and LLC cells (CRL-1642, ATCC) were cultured in Dulbecco’s modified Eagle’s medium high glucose (GIBCO, Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS). Cells were pre-treated with 10 μM AA or/and and 100 μM NG before they were stimulated with 5 ng/mL recombinant TGF-β1 (R&D Systems, MN, USA).

Wound-healing assay was performed when cell density reached about 80% confluence. Scratches were made with pipette tips and then cultured for another 24 h. The width of the wound was measured at 0, 6, 12, and 24 h after making the scratches and analyzed with ImageJ software (National Institutes of Health).

To perform migration assay, we starved LLC cells for 24 h before seeding them with serum-free medium in the 12-well transwell with 8.0 μm pore size (Corning, NY, USA). The transwell inserts were placed in receiver wells with medium containing 10% FBS and cultured for another 24 h. Non-migrated cells left in the transwell were swabbed before fixation and Giemsa staining. Then, the transwell membrane was cut and mounted for counting the migrated cells.

250 LLC cells were seeded in a 6-well plate and treated with AA or/and NG for colony-formation assay. When visible colonies appeared, cells were fixed with methanol for 10 min, followed by Giemsa staining. The number of colonies were then counted.

Establishment of mouse tumor models
Syngeneic melanoma and lung carcinoma model were established on C57BL/6 male mice (aged 8 weeks) via subcutaneous inoculation of 1 × 10⁶ B16F10 cells (CRL-6475, ATCC) or 2 × 10⁶ LLC cells (CRL-1642, ATCC). Mice were randomly divided into four groups when tumor size reached 50 mm² and AA (10 mg/kg), NG (50 mg/kg), or a combination of both was administered daily via i.p. injection as described previously. Mice were harvested 4 weeks after the start of treatment or when tumor size reached 2,000 mm³ and then the invasive and metastatic nodules were counted. All animal experiments were carried out by a protocol approved by Animal Ethics Experimental Committee (AEEC) at the Chinese University of Hong Kong.

Western blot analysis
Proteins from tumor tissue or cultured cells were extracted with ice-cold radioimmunoprecipitation assay (RIPA) buffer and subjected to SDS-PAGE and then electro-transfer to nitrocellulose membranes. After blocking with 5% BSA/Tris-buffered saline (TBS) buffer, membranes were incubated with primary antibodies against mouse p-Smad3, Smad7, p-ALK5 (Abcam, MA, USA), MMP2, MT1-MMP, TIMP2 (Merck Millipore, MA, USA), MMP9, MMP13, β-actin (Santa Cruz Biotechnology, CA, USA), and NF-κB p65 (Cell Signaling, MA, USA) dissolved in 1% BSA overnight at 4°C, followed by incubating with IRDye 800-conjugated secondary antibody (Rockland Immunocchemicals, PA, USA). Signals of target proteins were detected by Li-Cor/Odyssey infrared image system (LI-COR Biosciences, NE, USA) and then analyzed with ImageJ software (NIH, Bethesda, MD, USA).

RNA extraction and quantitative real-time PCR
Total RNA was extracted from tumor tissue or cultured cells with Pure-Link RNA Mini Kit (Life Technologies, NY, USA). RNA concentration was measured with Spectrophotometers Nanodrop (ND-2000, Thermo Fisher Scientific, MA, USA) and 1 μg total RNA was reverse transcribed into cDNA. Real-time PCR was performed with SYBR Green supermix on CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The ratio of target gene to GAPDH was calculated as Ratio = 2^ΔΔCt (GAPDH)-Ct (target). Primers used for real-time PCR are listed in Table 1.

Immunohistochemistry and immunofluorescence staining
Immunohistochemistry was performed on paraffin sections of LLC tumor tissue. After microwave-based antigen retrieval process, slides were incubated with primary antibodies including mouse MMP2 (Merck Millipore, MA, USA), p-Smad3, and Smad7 (Abcam, MA, USA) followed by horseradish peroxidase (HRP)-conjugated secondary antibodies, and then detected with DAB substrate. After immunostaining, sections were counterstained with hematoxylin. Due to the interference of melanin deposition, immunofluorescence staining was used to examine MMP2 expression level in melanoma tissue instead of immunohistochemistry. Periodate-lysine-paraformaldehyde (PLP)-fixed frozen sections were blocked with 5% BSA/PBS buffer, followed by overnight incubation of primary antibody against mouse MMP2 (Merck Millipore, MA, USA), and then 1 h incubation of Alexa 594-conjugated anti-rabbit secondary antibody. Slides were mounted with DAPI containing mounting medium and analyzed with fluorescence microscope (Leica Microsystems, Wetzlar, Germany).
ChIP assay
LLC cells with or without 3 h TGF-β1 stimulation were cross-linked with 37% formaldehyde. Then total chromatin was isolated with SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling, MA, USA). P-Smad3-DNA complexes were precipitated with anti-mouse p-Smad3 antibody (Abcam, MA, USA), while the p-p50-DNA complexes were precipitated with anti-mouse p-p50 antibody, and normal anti-rabbit IgG (Cell Signaling, MA, USA) was used as negative control. Targeted genomic regions within the precipitated DNA fragments were detected by PCR with the primers listed in Table 2 and then analyzed with gel electrophoresis.

Dual-luciferase reporter assay
A point mutation of the predicted Smad binding site (5'-AGA CTTCCC-3') on 5' UTR of MMP2 was introduced with DpnI (Promega, WI, USA) after linear amplification and then sequenced to confirm the desired mutation (5'-TCTGGAAGGAAA-3'). The primers used for amplification are forward: 5'- GCCCGCCCTTTTCCGCCCTTCTG TGGGAAAGGTTGGGTGCTGGGAGCTGCTTGCAT AT-3' and reverse: 5'-GTGGGAATGGTGGATGATCTCCTCAGGCACGCACACCC TCCAGAGATGCAGCGGAAAAGGCAGGGGC-3'. The original or point-mutated 5' UTR sequences of MMP2 were cloned into psi-CHECK2 Luciferase Reporter Vectors. The coding sequence of mouse Smad3 was also cloned into pcDNA3.1+

293T cells were transfected with the pGL3-MMP2 or pGL3-mutMMP2 plasmid using Lipofectamine 2000 (Invitrogen), and pcDNA3.1+-Smad3 plasmid was co-transfected into the cells to enhance the expression of mouse Smad3. The luciferase activities were analyzed by Dual-Luciferase Reporter Assay System (Promega, WI, USA).

Statistical analyses
Data were presented as mean ± standard deviation (SD). All data were analyzed with GraphPad Prism 8.4 software (San Diego, CA, USA) by one-way ANOVA for single variable analysis or two-way ANOVA for two independent variables analysis, followed by Tukey’s multiple comparisons post hoc test.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2021.01.006.

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AUTHOR CONTRIBUTIONS
G.-Y.L., Q.-M.W., and T.S.-K.M. performed experiments, analyzed data, and contributed to manuscript preparation. X.-R.H. participated in animal experiments and contributed to data analysis. X.-Q.Y. and H.-Y.L. designed and supervised the study and revised the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.
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