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Transforming growth factor-β1 induces invasion ability of HSC-4 human squamous cell carcinoma cells through the Slug/Wnt-5b/MMP-10 signaling axis

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Running title: TGF-β1 induces invasion through Slug/Wnt-5b/MMP-10 signaling

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Abbreviations[†]

Summary

Epithelial-mesenchymal transition (EMT) promotes the invasion of oral cancer cells, but the underlying molecular mechanisms are poorly understood. We previously demonstrated that transforming growth factor-β1 (TGF-β1) induces the expression of mesenchymal markers in human oral squamous cell carcinoma (hOSCC) HSC-4 cells. Intriguingly, the expression of the

[†]Abbreviations: BCA, bicinchoninic acid; CaMKII, calmodulin-dependent protein kinase II; Dvl, dishevelled; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; FBS, fetal bovine serum; FDZ, Frizzled; hOSCC, human oral squamous cell carcinoma; JNK, c-Jun N-terminal kinase; LRP, low-density lipoprotein receptor-related protein; MEF, myocyte enhancer factor; MEM, Eagle's minimum essential medium; MMP, matrix metalloproteinase; MS, Mass spectrometry; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction; RIPA, radio-immunoprecipitation assay; R-Smad, receptor-regulated Smad; SD, standard deviation; siRNA, small interfering RNA; TGF-β, transforming growth factor-β; TLC/LEF, T-cell factor/lymphoid enhancer-binding factor; TβR, transforming growth factor-β receptor; TβR-I, TGF-β receptor type I; TβR-II, TGF-β receptor type II; TIMP, tissue inhibitor of metalloproteinase.

EMT-related transcription factor Slug was also significantly upregulated upon TGF-β1 stimulation. However, the mechanism by which Slug transduces the TGF-β1-induced EMT signal to enhance the invasiveness of HSC-4 cells, is poorly understood. Proteomic analysis revealed that the expression of matrix metalloproteinase (MMP)-10 was upregulated in TGF-β1-stimulated cells. Additionally, a Boyden chamber assay revealed that the TGF-β1-induced increase in invasiveness of HSC-4 cells was significantly inhibited by MMP-10 siRNA. Intriguingly, Slug siRNA suppressed TGF-β1-induced expression of MMP-10. These results suggest that TGF-β1 induces invasion in HSC-4 cells through the upregulation of MMP-10 expression in a Slug-dependent manner. On the other hand, Slug siRNA suppressed TGF-β1-induced Wnt-5b expression. Wnt-5b significantly induced MMP-10 expression, while Wnt-5b siRNA suppressed the TGF-β1-induced increase in invasiveness, suggesting that TGF-β1-induced expression of MMP-10, and the resulting upregulation of invasiveness, are mediated by Wnt-5b. Overall, these results suggest that TGF-β1 stimulates HSC-4 cell invasion through the Slug/Wnt-5b/MMP-10 signaling axis.

Key words: invasion; MMP-10; Slug; TGF-β; Wnt-5b

Human oral squamous cell carcinoma (hOSCC) is a common type of cancer (*1, 2*). Despite the development of advanced treatment, patients suffering from hOSCC are still faced with poor prognosis and high mortality rates. Especially the mechanisms of hOSCC invasion and metastasis are not well understood (*3*). Intriguingly, gene expression profiling shows that epithelial-mesenchymal transition (EMT) is a characteristic feature of high-risk head and neck squamous cell carcinoma (*2-4*). EMT is a process that allows a polarized epithelial cell to assume a mesenchymal cell phenotype, which is characterized by enhanced motility and invasiveness (*5*). In addition, EMT also causes the disruption of cell-cell adherence, loss of apico-basal polarity, and matrix remodeling, and thereby promotes tumor metastasis (*6, 7*). At the molecular level, EMT is characterized by the loss of epithelial markers such as E-cadherin, down-regulation of cytokeratins, up-regulation of mesenchymal markers such as N-cadherin and vimentin, and acquisition of a fibroblast-like motile and invasive phenotype (*8*). However, the molecular mechanisms underlying hOSCC invasion associated with EMT are not well characterized.

TGF-β exerts multiple functions by binding to transmembrane serine/threonine kinases, TGF-β receptor type I (TβR-I) and type II (TβR-II), which mediate specific intracellular signaling

pathways through the phosphorylation of receptor-regulated Smads (R-Smads). Phosphorylated R-Smads associate with Smad4 and translocate to the nucleus where they control the transcription of target genes in cooperation with other transcription factors and transcriptional co-activators or co-repressors (*9, 10*). TGF-β is associated with the malignant transformation and aggravation of cancer through the induction of EMT (reviewed in *11-13*). Many transcriptional factors, such as Snail (*14*) and Slug (*15*), participate in EMT (*16, 17*) , and the interaction between Smads and EMT-promoting transcriptional factors results in the formation of complexes that engage in both the repression of epithelial genes and the activation of mesenchymal genes.

Recent studies on tumor biology revealed that complex interactions of tumor cells with their adjacent microenvironment, including the extracellular matrix (ECM), are necessary for various mechanisms involved in tumor development and progression (reviewed in *18*). Thus, the interaction between tumor cells and the ECM controls most aspects of tumorigenicity, including EMT and subsequent tumor cell invasion. Several matrix metalloproteinases (MMPs), including MMP-2 and MMP-9, also play a role in tumor invasion and metastasis (*19-21*). In addition, TGF-β promotes tumor invasion by stimulating the expression of proteases that digest ECM components. Several types of MMPs are upregulated by TGF-β stimulation, such as MMP-1, -3,

-9, and -10 (*22, 23*). MMP-10 (EC3.4.24.22), also known as stromlysin-2, targets a broad range of extracellular matrix proteins, including collagen type IV, gelatin, elastin, fibronectin, laminin, and proteoglucan, as well as proMMP-1, -7, -8, -9, and -13 (*24*). In particular, ectopic overexpression of MMP-10 induces invasion of hOSCC cells (*25*).

The Wnt signaling pathway is important for cell development and cancer (*26, 27*). The Wnt family of glycoproteins consists of 19 Wnt ligands in humans (*28*). Wnt ligands bind to membrane-bound receptors, including 10 types of Frizzled (FDZ) receptors, low-density lipoprotein receptor-related protein (LRP) 5/6, and atypical receptor tyrosine kinases (RTKs)-PKT7, ROR2, and RYK (*29, 30*). Wnt signaling can roughly be divided into two pathways: the β-catenin dependent “canonical” and the β-catenin independent “non-canonical” pathway. In canonical signaling, binding of Wnt ligands to both an FDZ receptor and co-receptor (LRP5/6) leads to the activation of intracellular signaling cascades through the dissociation of cytoplasmic β-catenin from membrane. Then, cytoplasmic β-catenin binds to T-cell factor/lymphoid enhancer-binding factor (TLC/LEF) and activates target gene transcription. The EMT-induced disruption of E-cadherin-mediated adherens junctions also causes the translocation of β-catenin from the cell membrane into the cytoplasm, allowing it to enter the nucleus and

transactivate genes via the canonical Wnt signaling pathway (*31-33*). Thus, Wnt/β-catenin activation is an integral part of EMT (*34, 35*). It has been shown that β-catenin is predominantly localized in the nucleus in human oral squamous cell carcinoma (hOSCC) cells at the invasive front by Wnt3 (*36*). On the other hand, other types of Wnt ligands bind to their respective receptors and elicit cellular responses. Wnt-5a (*37*) and b (*25*) are the ligands for the activation of the non-canonical signaling pathway. Wnt-5a signaling is stimulated by TGF-β and regulates ECM production in airway smooth muscle cells (*37*). This signaling pathway consists of the Ca^{2+}-dependent signaling and planar cell polarity pathways. In the Ca^{2+} pathway, Ca^{2+}-dependent molecules, such as calcineurin, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and PKC are activated. The planar cell polarity pathway involves the activation of small Rho-GTPases and c-Jun N-terminal kinases (JNKs) or the Rho-kinase signaling (*29, 38*). Intriguingly, Deraz et al. previously reported that Wnt-5b promotes the upregulation of MMP-10 in hOSCC (*25*).

We previously demonstrated that TGF-β1 promotes the EMT of hOSCC cells (*39*). More specifically, TGF-β1 upregulates the expression levels of mesenchymal markers, such as N-cadherin, vimentin, and integrin α3β1-targeted proteins, and also enhances cell migratory activity in HSC-4, a hOSCC cell line. Intriguingly, the expression level of the EMT-related

transcription factor Slug was also significantly upregulated upon TGF-β1 stimulation, suggesting that Slug may control the EMT of HSC-4 cells stimulated with TGF-β1. However, it remains to be clarified how Slug, the different Wnt family members, and MMPs cooperate in the transduction of TGF-β-induced signals to stimulate the invasion ability of hOSCC cells. In this paper, we discuss the functional relationship between Slug, MMP-10, and Wnt-5b with regard to the upregulation of HSC-4 cellular invasion in response to TGF-β stimulation.

Materials and Methods

Materials

Cultured cell lines were obtained from the Human Science Resource Cell Bank (Osaka, Japan). Recombinant human TGF-β1 was purchased from PEPROTECH (Rocky Hill, NJ, USA). The c-JNK inhibitor SP600125 was provided by Merck-Millipore (Frankfurt, Germany). Dvl-PDZ Domain Inhibitor II, which disrupts FZD-dishevelled (Dvl) interactions in Wnt signaling, was also purchased from Merck-Millipore. Human recombinant DKK1 protein, which inhibits

non-canonical Wnt signaling by preventing LRP5/6 interaction with Wnt, was provided by ATGen (Seongnam-si, South Korea). Recombinant human Wnt-5b was purchased from R&D systems (Minneapolis, MN, USA). Protease inhibitor cocktail for use with mammalian cell and tissue extracts and phosphatase inhibitor cocktail 1 and 2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were analytical grade.

Cell culture

All cell lines were grown at 37°C and 5% CO_2. Human HSC-2 and HSC-4 squamous cell carcinoma cells were cultured in Eagle's minimum essential medium (MEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Rockville, MD, USA). HSC-3 cells were cultured in DMEM (Gibco BRL) containing 10% FBS. SAS cells were cultured in PRIM1640 medium (Gibco BRL) supplemented with 10% FBS. The culture medium was removed and replaced with serum-free medium 24 h prior to TGF-β1 stimulation. In the experiments pertaining to the production of secreted proteins, such as MMP-10 and Wnt-5b, 4×10^5 cells (HSC-4) were cultured in 6-well plates for 48 or 72 h with 3.0 mL serum-free medium, containing 10 ng/mL TGF-β1. A fraction of the conditioned medium (500 μL) was harvested and

then concentrated by ultrafiltration using Microcon-10 filters (cut-off, 10 kDa; Merck) to a volume of 20 µL. An equal volume of sample buffer (Laemmli 2× concentrate; Sigma-Aldrich) was added to the concentrated medium and the samples were separated by SDS-PAGE. Based on our previous work (*39*), we compared the expression of ECM proteins in conditioned medium for an equal number of cells, without detection of a loading control (Supplementary Fig. S1C).

Mass spectrometry analysis

SDS-PAGE was carried out on a 10–20% acrylamide gradient gel (ATTO Co., Tokyo, Japan). Protein bands were stained with Flamingo fluorescent gel stain (BIO-RAD, Hercules, CA, USA). Digestion of proteins in the gel pieces was carried out according to a previously described method (*39*). The peptides were eluted with a gradient of 10–65% acetonitrile in 0.1% formic acid by capillary HPLC (Agilent 1100 System; Agilent). Mass spectrometry (MS) was carried out on an HCT ultra si (Bruker Daltonics, Bremen, Germany), according to the manufacturer's instructions. Protein sequence database searches were performed with Mascot (Matrix Science, Boston, MA, USA) using the MS/MS peptide ions.

Quantitative real time RT-PCR (qRT-PCR)

For total RNA preparation, 1×10^5 cells were cultured in 24-well tissue culture plates. Total RNA was isolated using the ISOGEN reagent (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. The first-strand cDNA was prepared from 4 μg of total RNA using the poly-A primer provided in the RT-PCR System Kit (Takara Bio Inc., Shiga, Japan). qRT-PCR was performed on a Thermal Cycler Dice Real Time System (Takara Bio) using SYBR Premix Ex Taq II (Takara Bio) with gene-specific primers (listed in Table I). The mRNA expression levels of the target genes were normalized to those of the endogenous reference gene β-actin and are shown in terms of fold increase or decrease relative to the level of the control sample.

Western blot analysis

A total of 5×10^5 cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease and phosphatase inhibitors (Sigma-Aldrich). The protein content of the samples was measured using BCA reagent (Pierce, Rockford, IL, USA). To examine marker proteins in cell lysate, 4×10^5 cells were cultured in a 6-well plate in serum-free MEM with or without 10 ng/mL TGF-β1 for the indicated

times. The cells were dissolved in SDS sample buffer containing protease and phosphatase inhibitors (Sigma-Aldrich). Acrylamide gels of 12.5% (ATTO Co., Tokyo, Japan) were used for protein separation via SDS-PAGE, and the proteins were subsequently transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were probed with the following primary antibodies: anti-MMP-10 mouse (LA-12) and anti-MMP-10 goat (I-18) antibody (Fig. 1C, 2E, 3C, and 4B) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-pJNK rabbit antibody (#9251; Cell Signaling, Beverly, MA, USA) (Fig. 6A and B), anti-JNK rabbit antibody (#9252; Cell Signaling) (Fig. 6A and B), and anti-Wnt-5b rabbit antibody (ab124818; Abcam, Cambridge, UK) (Fig. 5C), while an anti-β-actin mouse antibody (clone C4; Santa Cruz) was used as a loading control in the experiments (Fig. 6A, B and E). Anti-Slug (A-7; Santa Cruz) antibody was also used for western blotting (Fig. 3A and 5B) to confirm Slug knockdown at the protein level. All blots were incubated with alkaline phosphatase-conjugated secondary antibody and signals were detected using an alkaline phosphatase substrate kit (BCIP/NBT Substrate Kit; Vector Laboratories Inc., Burlingame, CA, USA).

Suppression of gene expression by small interfering RNA (siRNA)

The sense sequence of human Slug siRNA (MISSION siRNA, Sigma-Aldrich) was previously described (*39*). The siRNAs of MMP-10 (Stealth™ RNAi, MMP10HSS106682) and Wnt5b (Stealth™ RNAi, WNT5BHSS129775) were purchased from Life Technologies (Carlsbad, CA). Logarithmically growing cells were seeded at a density of 1×10^5 cells per well in a 24-well tissue culture plate and transfected with 10 nM siRNA using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were stimulated using 10 ng/mL TGF-β1 and subsequently used for qRT-PCR analysis of vimentin gene expression or in an invasion assay as described below. Stealth™ RNAi Negative Control Low GC Duplex (Life Technologies) was used as a negative control. Suppression of gene expression by siRNA was evaluated by qRT-PCR and western blotting analyses for the targeted molecules.

Cell invasion assay with a Boyden chamber

Polycarbonate membranes coated with Matrigel were used as the insert of the Boyden chamber for invasion assays (Corning Inc., Corning, NY, USA). First, the cells were transfected with siRNA as described above. Then, they were treated with 10 ng/mL TGF-β1 under serum-free

conditions with or without reagents, such as SP600125, for 72 h. Subsequently, the cells were plated in the upper compartment of a Boyden chamber apparatus at a density of 1×10^5 cells in serum-free medium with or without inhibitor. Cells were allowed to migrate into medium containing 10% FBS in the lower chamber for 24 h at 37°C. Following the 24-h incubation, the filter was fixed in 4% paraformaldehyde and stained with hematoxylin (Wako Pure Chem. Ind., Osaka, Japan) for 16 h. Cells that had migrated onto the lower side of the membrane were counted. The values are averages of experiments conducted in triplicate.

Statistical analysis

At least 3 independent replicates were performed for all experiments. The results are expressed as the mean ± standard deviation (SD). Data were analyzed using the unpaired Student's *t*-test. In all statistical analyses, a P value < 0.05 was considered statistically significant, and all P values are two-sided.

Results

TGF-β1 upregulates MMP-10 expression in HSC-4 cells

We examined which proteins are involved in cell invasion by performing a proteomic analysis on the conditioned media from HSC-4 cells stimulated with TGF-β1 (Fig. 1A and 1B). We found that MMP-1 and -10 were highly expressed in conditioned media upon TGF-β1 stimulation, than that in the control media. However, the expression levels of other MMPs were not significantly increased. The increase in MMP-10 protein expression induced by TGF-β1 stimulation was confirmed by western blotting (Fig. 1C). MMP-10 was of particular interest because this protease digests type IV collagen, an important constituent of the basal membrane that blocks tumor cell invasion into the fibrous tissue under the epithelial tissue, and activates other MMPs (*24*). We confirmed the increase in MMP-10 expression upon stimulation with TGF-β1 at the mRNA level in HSC-4 cells via qRT-PCR analysis (Fig. 1D). HSC-4 cells show a strong upregulation in MMP-10 expression in response to TGF-β1 stimulation, while other hOSCC cells, including HSC-2, HSC-3, and SAS cells, did not (Fig. 1D).

TGF-β1 promotes invasion ability of HSC-4 cells through expression of MMP-10

To understand whether MMP-10 expression affects the invasiveness of HSC-4 cells, we used

siRNA to knockdown MMP-10. First, we confirmed that MMP-10 siRNA indeed reduced MMP-10 expression at the mRNA level, using qRT-PCR (Fig. 2A), and at the protein level, via western blot analysis (Fig. 2B). Intriguingly, MMP-10 siRNA inhibited the TGF-β1-induced increase in HSC-4 invasion ability (Fig. 2C). These results suggest that the TGF-β1-induced increase in invasion ability of HSC-4 cells is mediated by MMP-10 expression.

TGF-β1 upregulates MMP-10 expression in a Slug-dependent manner

We next examined whether Slug affected the TGF-β1-induced expression of MMP-10 and the invasion ability of HSC-4 cells. Therefore, we downregulated Slug using siRNA, which was previously demonstrated to downregulate the expression of Slug in HSC-4 cells at both the mRNA and protein level (*39*). We also confirmed the efficient downregulation of Slug in this study (data not shown). The TGF-β1-induced expression of MMP-10 was significantly downregulated in HSC-4 cells by administration of Slug siRNA as compared to that of control siRNA at the mRNA (Fig. 3A) and protein (Fig. 3B) level. Intriguingly, Slug siRNA significantly suppressed the TGF-β1-induced invasion ability of HSC-4 cells while control siRNA did not (Fig. 3C). These results indicate that TGF-β1 stimulates MMP-10 expression in HSC-4 cells in a

Slug-dependent manner.

Taking into account our observations in Figures 1 and 2, we propose that Slug-dependent induction of MMP-10 expression by TGF-β1 plays an important role in the regulation of the invasion ability of HSC-4 cells.

TGF-β1-induced MMP-10 expression seems to be mediated through non-canonical Wnt signaling possibly activated by Wnt-5b

In order to investigate whether TGF-β1 affects canonical or non-canonical Wnt signaling in HSC-4 cells, we evaluated the mRNA expression levels of different players in the canonical (Wnt-3a), and non-canonical (Wnt-5a, and -5b) pathway after TGF-β1 stimulation. TGF-β1 significantly upregulated Wnt-5b expression (Fig. 4A), but did not affect Wnt-3a (Fig. 4B) and Wnt-5a (Fig. 4C) expression. In addition, a universal Wnt signal inhibitor, Dvl-PDZ domain inhibitor II, significantly suppressed the TGF-β1-induced expression of MMP-10 mRNA (Fig. 4D). On the other hand, DKK-1 inhibitor, a protein that binds co-receptor LRP5/6 and specifically inhibits canonical Wnt signaling, did not affect the expression levels of MMP-10 in HSC-4 cells stimulated with TGF-β1 (Fig. 4E). We also confirmed that recombinant human Wnt-5b

(rhWnt-5b) significantly upregulated the expression of MMP-10 in HSC-4 cells (Fig. 4F), as previously described (*25*).

These data suggest that non-canonical Wnt signaling, possibly activated by Wnt-5b, mediates the induction of MMP-10 expression by TGF-β1.

TGF-β1 induces Wnt-5b expression through Slug, which increases the invasiveness of HSC-4 cells

The TGF-β1-induced expression of Wnt-5b was significantly downregulated by Slug siRNA in HSC-4 cells at both the mRNA (Fig. 5A) and protein (Fig. 5B) level. These results suggest that TGF-β1 induces the expression of Wnt-5b in a Slug-dependent manner. In addition, HSC-4 cells in which Wnt-5b was downregulated by siWnt-5b (Fig. 5C), exhibited a decrease in invasion ability in our Boyden chamber assay upon TGF-β1 treatment (Fig. 5D).

Wnt-5b induces MMP-10 expression and invasion in HSC-4 cells in a JNK-dependent manner

In order to gain more insights into the molecular mechanisms underlying the TGF-β1-induced promotion of invasion in HSC-4 cells, we evaluated the role of JNK, which is known to be a key

mediator of Wnt-5b-induced signal transduction (*25*), in this process. Firstly, JNK was phosphorylated after 1 h of TGF-β1 stimulation in HSC-4 cells (Fig. 6A). We also found that rhWnt-5b induced JNK phosphorylation in HSC-4 cells after 15 min of stimulation (Fig. 6B). The JNK inhibitor SP600125 significantly suppressed the TGF-β1-induced expression of MMP-10 but not that of Slug and Wnt-5b at mRNA level (Fig. 6C). The SP600125 suppressed the TGF-β1-induced expression of MMP-10 but not that of Slug at protein level (Fig. 6D). Finally, SP600125 clearly suppressed the TGF-β1-induced invasion ability of HSC-4 cells (Fig. 6E).

Discussion

Our data support a model in which the transcription-related factor Slug induces Wnt-5b expression upon TGF-β1 stimulation (Fig. 4A and 5B). Wnt-5b in turn activates the non-canonical Wnt pathway in HSC-4 cells through autocrine and/or paracrine signaling, and subsequently upregulates MMP-10 expression (Fig. 4) in a JNK-dependent manner (Fig. 6). In summary, we propose that the expression level of MMP-10 is increased by TGF-β1 treatment in HSC-4 cells through a Slug/Wnt-5b/JNK signaling axis. On the other hand, the invasiveness of

HSC-4 cells was dependent on the expression level of MMP-10, as downregulation of MMP-10 clearly decreased invasion in HSC-4 cells (Fig. 2C). In addition, Wnt-5b siRNA downregulated the TGF-β1-induced invasion ability of HSC-4 cells (Fig. 5D), suggesting that TGF-β1 indirectly affects HSC-4 cell invasion through the expression of Wnt-5b and MMP-10. Thus, TGF-β1 stimulates the invasion ability of HSC-4 cells through the Slug/Wnt-5b/JNK/MMP-10 signaling axis.

Slug is an important transcription factor in EMT, as it regulates the expression of EMT-related genes (*15, 40*). We actually showed that Slug induced the expression of vimentin, but not of N-cadherin, during EMT in HSC-4 cells (*39*). It has not been elucidated whether Slug regulates the expression of MMP-10 and Wnt-5b in hOSCC cells. In this paper, we demonstrate for the first time that Slug stimulates the expression of both MMP-10 (Fig. 3A and B) and Wnt-5b (Fig. 5A and B) in hOSCC cells.

There are a few reports that have shown the upregulation of Wnt-5b expression in tumor cells stimulated by TGF-β1, for example in human pituitary tumor cells (*41*), however, the function of Wnt-5b in cancer remains poorly understood. In contrast, the function of Wnt-5a in cancer has been relatively well investigated (reviewed in *42*). Several reports correlate increased Wnt-5a

expression with decreased patient survival and increased invasion ability in melanoma, gastric, ovarian, and colorectal cancer (*42, 43*). In addition, Kumawat *et al*. reported that TGF-β1 induced the expression of Wnt-5a, but not Wnt-5b, in airway smooth muscle cells (*37*).

There are also a few reports that demonstrate the upregulation of MMP-10 expression during TGF-β1-induced EMT (*44, 45*). TGF-β1 plus EGF stimulation induces expression of MMP-10 and MMP-1 in HaCaT II-4 keratinocytes and promotes the invasion ability of cells growing on collagen type I gels (*44*), presumably through enhanced collagen degradation. Another report showed that the expression of MMP-10 is upregulated by TGF-β through myocyte enhancer factor (MEF)-2A in mouse and human mammary epithelial cells (NMuMG and MCF10A, respectively) (*45*).

It was previously reported that MMP-10 induces tumor progression and invasion in cervical tumors (*46*) and in hOSCCs (*47*). We compared the invasiveness of 3 cell lines derived from hOSCCs (HSC-2, HSC-4, and SAS) after TGF-β1 stimulation and found that TGF-β1 promoted the invasion in HSC-4 cells and SAS cells, but not in HSC-2 cells (data not shown). In addition, as shown in Fig. 1D, MMP-10 expression in HSC-4 cells was significantly induced by TGF-β1, whereas in HSC-2, HSC-3, and SAS cells, this was not the case. These results suggest that other

factors besides MMP-10 also play important roles in the TGF-β1-induced increase in invasion in hOSCC cells.

As MMP-2 and MMP-9 are also involved in invasion and metastasis (*19-21*), we evaluated expression of other MMPs besides MMP-1 and MMP-10 in HSC-4 cells. MMP-2 and MMP-9 expression levels were upregulated 24 and 48 h after TGF-β1 stimulation (Supplementary Fig. S1 A and B). In addition, expression of MMP-2 was more strongly induced by TGF-β1 than that of MMP-9 (Supplementary Fig. S1A and B). However, MMP-14 expression levels were not changed in HSC-4 cells upon TGF-β1 treatment (data not shown). The roles of MMP-1, MMP-2, and MMP-9 in the invasion ability of TGF-β1-stmimulated HSC-4 cells should be elucidated in future studies.

The protein levels of MMP-10, as a secreted form in the medium used for HSC-4 cell culture and as a non-secreted form in the cell lysates, were evaluated by western blot. TGF-β1 treatment induced a remarkable upregulation of MMP-10 protein expression and almost all protein was secreted into the medium (Supplementary Fig. S1C). MMP-1 protein levels were lower than that of MMP-10 (Fig. 1A), suggesting that MMP-10 is a major MMP in TGF-β1-stimulated HSC-4. Intriguingly, the expression levels of TIMP1 and TIMP2, two MMP inhibitors, were significantly

downregulated 24 h after TGF-β1 stimulation (data not shown). This suggests that the TGF-β1-induced increase in invasion in HSC-4 cells is reciprocally controlled by the upregulation of MMPs and the downregulation of TIMPs.

While it was previously suggested that increased MMP-10 expression in hOSCC depends on the pathologic processes and the invasion abilities of tumors (*47*), our findings show that the invasion ability of the cells also depends on the expression level of MMP-10. A specific MMP-10 inhibitor could be valuable as a research tool and for clinical applications in hOSCC. However, to date, a specific and useful inhibitor of MMP-10 has not been developed. Our observations on the molecular mechanisms that regulate invasion in HSC-4 cells led to the identification of a Slug/Wnt-5b/JNK/MMP-10 signaling axis, which can aid in finding novel therapeutic molecular targets to inhibit invasion of cancer cells.

Supplementary Data

Supplementary data are available at JB Online.

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Conflict of Interest

None declared.

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Figure legends

Fig. 1. TGF-β1 induces expression of MMP-10 in HSC-4 cells

(A) HSC-4 cells were treated with 10 ng/mL TGF-β1. The secreted proteins, present in the culture medium, were separated via SDS-PAGE and analyzed by LC-MS/MS. Proteomic analysis identified MMP-1 and MMP-10 (bands indicated by arrows) in the TGF-β1-stimulated cells. (B) The mass data were analyzed using Mascot software against a protein database for protein identification. (C) The conditioned medium was also analyzed by western blotting with anti-MMP-10 antibodies (LA-12 clone). (D) The expression of MMP-10 in four TGF-β-stimulated hOSCC cell lines was examined by qRT-PCR. The values have been normalized to the β-actin mRNA level. Data represent the mean ± SD of triplicate experiments (*$P < 0.05$; **$P < 0.01$).

Fig. 2. TGF-β1 promotes invasion in HSC-4 cells through MMP-10 expression

(A) HSC-4 cells were transfected with MMP-10 siRNA (siMMP-10) or control siRNA (siControl). The expression of MMP-10 mRNA was examined by qRT-PCR. The values have

been normalized to the β-actin mRNA levels. Data represent the mean ± SD of triplicate experiments (*$P < 0.05$; **$P < 0.01$). (B) For the detection of MMP-10 protein, culture media were collected 72 h after TGF-β1 (10 ng/mL) stimulation, and then subjected to western blotting analysis. (C) First, the cells were transfected with control siRNA (black bars) or MMP-10 siRNA (gray bars). Then, the invasiveness of the cells was examined in a Boyden chamber assay. Data represent the mean ± SD of 3 technical replicates for each time point (*$P < 0.05$; **$P < 0.01$).

Fig. 3. TGF-β1 induces MMP-10 expression in a Slug-dependent manner

(A) HSC-4 cells were transfected with Slug siRNA (siSlug) or control siRNA (siControl). The expression of MMP-10 mRNA was examined by qRT-PCR. (B) The protein expression levels of MMP-10 were determined by western blotting with anti-MMP-10 (LA-12) antibodies. (C) The invasiveness of HSC-4 cells was evaluated in a Boyden chamber assay after transfection with siSlug (light gray bars) or siControl (dark gray bars). After a 24-h incubation, the penetrated cells on the lower side of the membrane were stained by hematoxylin. Data represent the mean ± SD of 3 technical replicates for each time point (*$P < 0.05$; **$P < 0.01$).

Fig. 4. TGF-β1-induced MMP-10 expression seems to be mediated through non-canonical Wnt signaling possibly activated by Wnt-5b

HSC-4 cells with (gray bars) or without (black bars) stimulation with 10 ng/mL TGF-β1 for 24 or 48 h in serum-free medium were analyzed by qRT-PCR. The expression levels of (A) Wnt-5b, (B) Wnt-3a, and (C) Wnt-5a were examined. (D and E) HSC-4 cells with or without a 48-h stimulation using 10 ng/mL TGF-β1 were treated with (D) Dvl-PDZ Domain Inhibitor II (10 μM) or (E) DKK-1 (10 μg/mL) 60 min prior to TGF-β1 treatment. The expression levels of MMP-10 were examined by qRT-PCR. (F) HSC-4 cells were stimulated with 0, 50, or 100 ng/mL rhWnt-5b for 24 h in serum-free medium. The expression levels of MMP-10 were assessed by qRT-PCR. Data represent the mean ± SD of triplicate for each time point (*$P < 0.05$; **$P < 0.01$).

Fig. 5. TGF-β1 increases the invasiveness of HSC-4 cells by induction of Wnt-5b expression through Slug

(A) HSC-4 cells were transfected with Slug siRNA (siSlug) or control siRNA (siControl). The expression of Wnt-5b mRNA was examined by qRT-PCR. (B) The protein expression levels of Wnt-5b were determined by western blotting using with anti-Wnt-5b (ab124818) antibodies. (C)

The cells were transfected with Wnt-5b siRNA (siWnt-5b) or siControl and mRNA expression of Wnt-5b was examined by qRT-PCR. (D) The invasiveness of HSC-4 cells transfected with siWnt-5b (light gray bars) or siControl (dark gray bars) was evaluated in a Boyden chamber assay. After 24 h of incubation, the penetrated cells on the lower side of the membrane were stained by hematoxylin. Data represent the mean ± SD of 3 technical replicates for each time point (*P < 0.05; **P < 0.01).

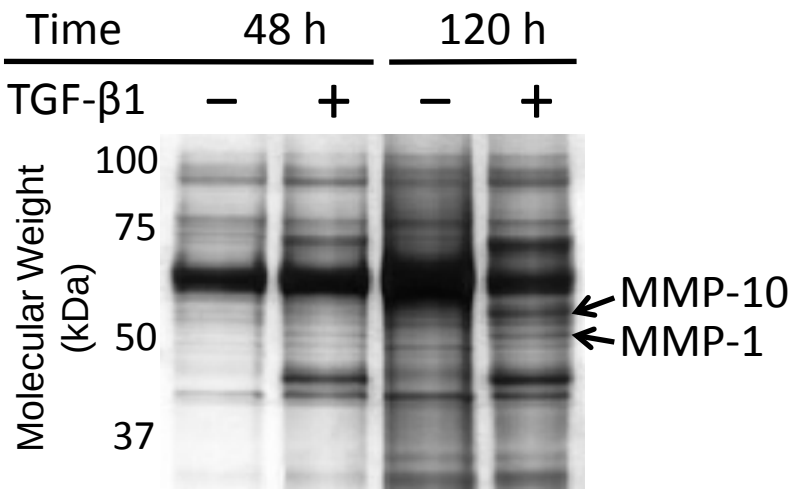
Fig. 6. Wnt-5b induces MMP-10 expression and invasion in HSC-4 cells in a JNK-dependent manner

(A) TGF-β1-induced phosphorylation of JNK in HSC-4 cells was examined by western blotting with anti-phosphoJNK and anti-JNK antibodies. Whole-cell extracts were prepared from cells treated with 10 ng/mL TGF-β1. The level of β-actin was used as a loading control. (B) rhWnt-5b-induced phosphorylation of JNK in HSC-4 cells was examined by western blotting with anti-phosphoJNK and anti-JNK antibodies. β-actin was used as a loading control. (C and D) HSC-4 cells were treated with SP600125 (10 μM) or DMSO as a negative control, 60 min prior to TGF-β1 treatment. (C) The expression levels of target genes were examined by qRT-PCR. (D)

The protein expression levels of MMP-10 and Slug were determined by western blotting using anti-MMP-10 (I-18) and anti-Slug antibodies. β-actin was used as a loading control. (E) The invasiveness of HSC-4 cells in the presence of SP600125 (light gray bars) or DMSO as a control (dark gray bars) was evaluated in a Boyden chamber assay. After 24 h of incubation, the penetrated cells on the lower side of the membrane were stained by hematoxylin. Data represent the mean ± SD of 3 technical replicates for each time point (*$P < 0.05$; **$P < 0.01$).

Figure 1.

A



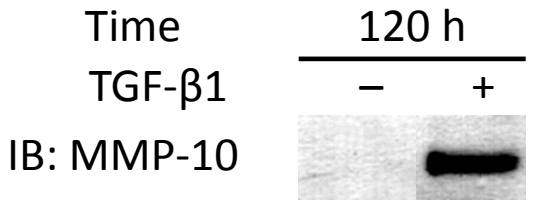
B

TGF-β1-induced MMPs identified by mass spectrometry

Protein Name	Pro-form MW (kDa)	No. of matched peptides[a]	Mascot score	Sequence coverage (%)
MMP-1	52	16	339	8
MMP-10	56	13	259	31

[a]Matched peptides include all peptides that differ either by sequence, modification, or charge.

C



D

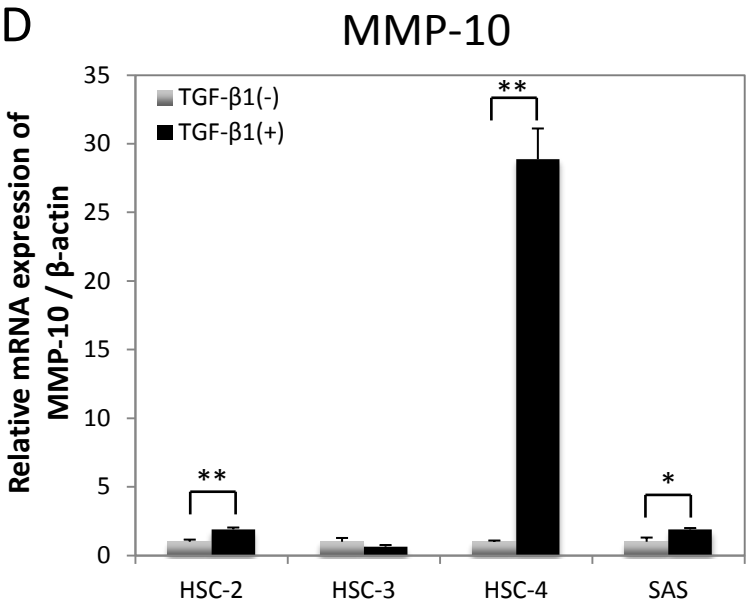
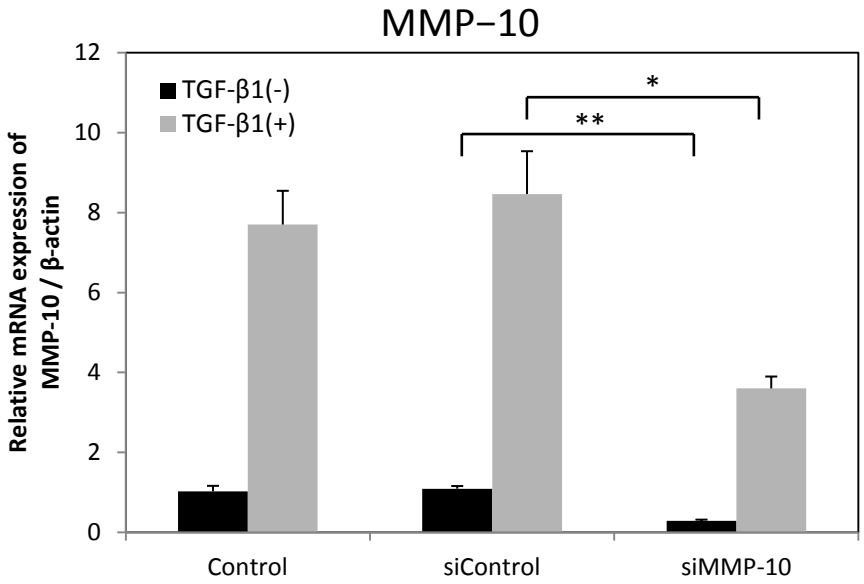
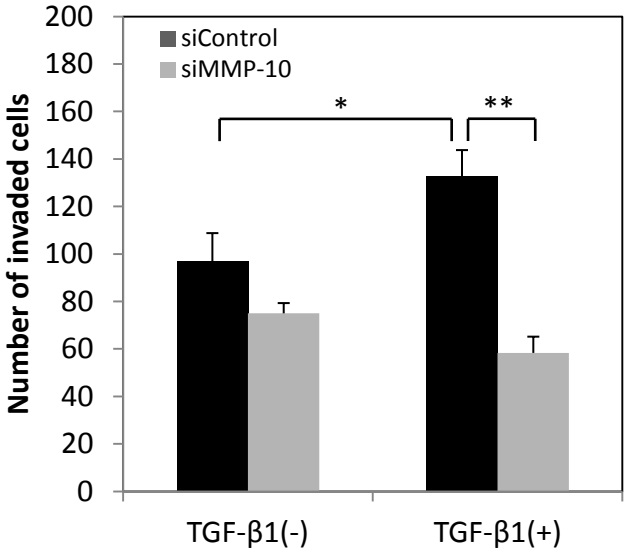


Figure 2.

A


MMP−10
Relative mRNA expression of MMP-10 / β-actin
TGF-β1(-)
TGF-β1(+)
12
10
8
6
4
2
0
*
**
Control
siControl
siMMP-10

C


Number of invaded cells
siControl
siMMP-10
200
180
160
140
120
100
80
60
40
20
0
*
**
TGF-β1(-)
TGF-β1(+)

B

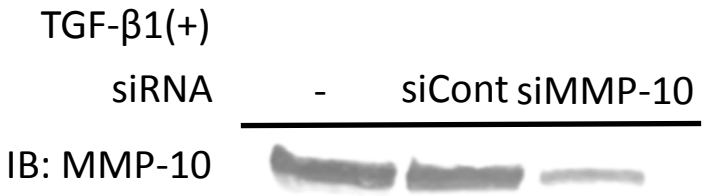
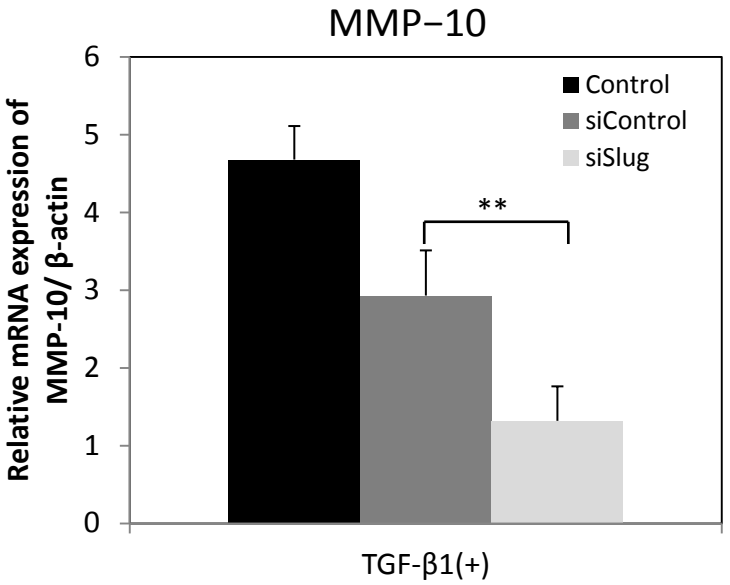
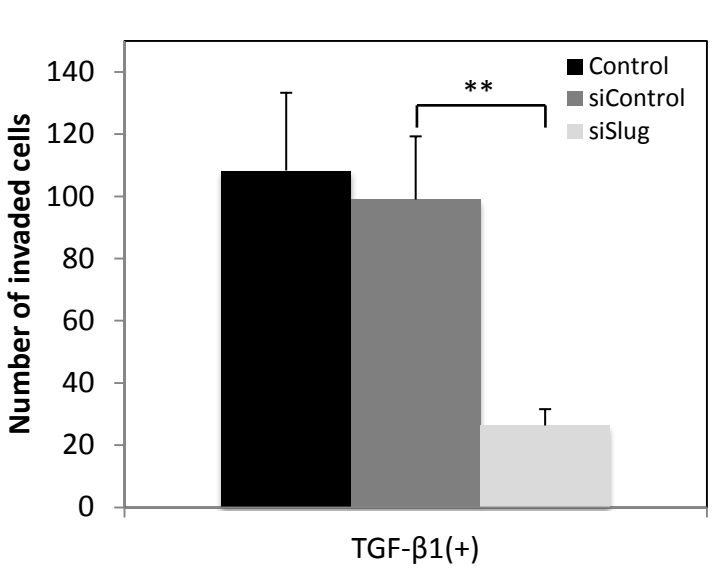

TGF-β1(+)
siRNA
-
siCont
siMMP-10
IB: MMP-10

Figure 3.

A


MMP−10
Relative mRNA expression of MMP-10/ β-actin
6
5
4
3
2
1
0
Control
siControl
siSlug
**
TGF-β1(+)

C


Number of invaded cells
140
120
100
80
60
40
20
0
Control
siControl
siSlug
**
TGF-β1(+)

B

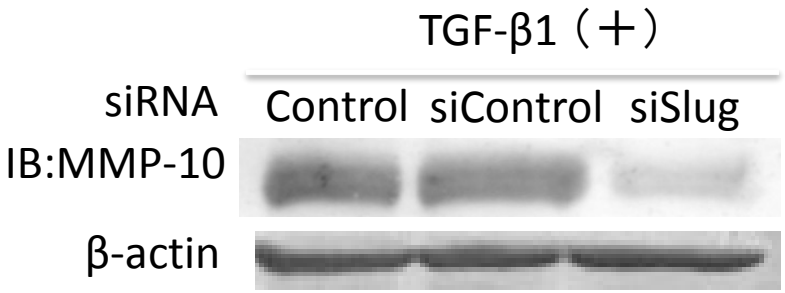

TGF-β1（＋）
siRNA
Control siControl siSlug
IB:MMP-10
β-actin

Figure 4.

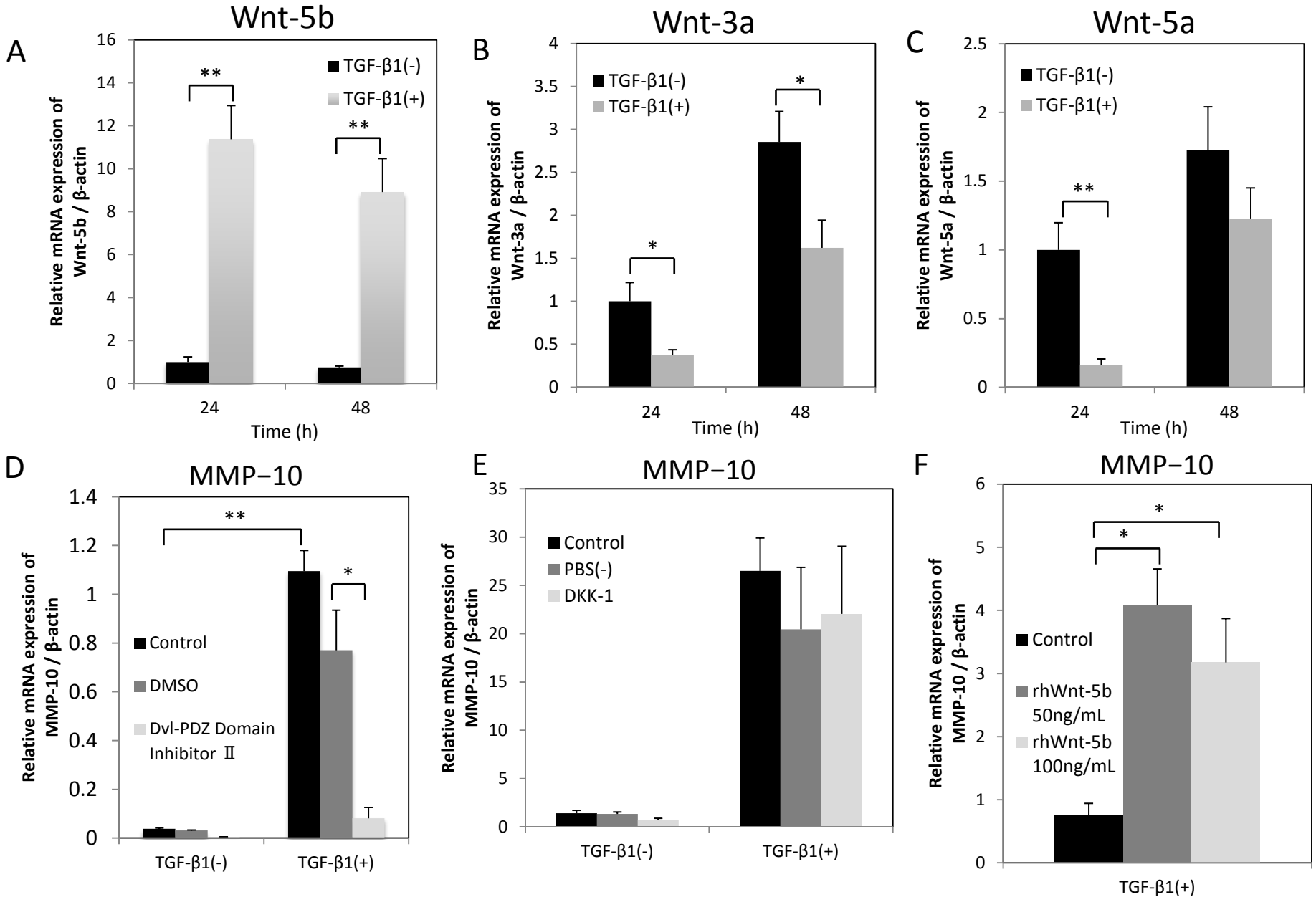

A
Wnt-5b
Relative mRNA expression of Wnt-5b / β-actin
TGF-β1(-)
TGF-β1(+)
**
**
Time (h)
24
48
B
Wnt-3a
Relative mRNA expression of Wnt-3a / β-actin
TGF-β1(-)
TGF-β1(+)
*
*
Time (h)
C
Wnt-5a
Relative mRNA expression of Wnt-5a / β-actin
TGF-β1(-)
TGF-β1(+)
**
Time (h)
D
MMP−10
Relative mRNA expression of MMP-10 / β-actin
**
*
Control
DMSO
Dvl-PDZ Domain Inhibitor Ⅱ
TGF-β1(-)
TGF-β1(+)
E
MMP−10
Relative mRNA expression of MMP-10 / β-actin
Control
PBS(-)
DKK-1
TGF-β1(-)
TGF-β1(+)
F
MMP−10
Relative mRNA expression of MMP-10 / β-actin
*
*
Control
rhWnt-5b 50ng/mL
rhWnt-5b 100ng/mL
TGF-β1(+)

Figure 5.

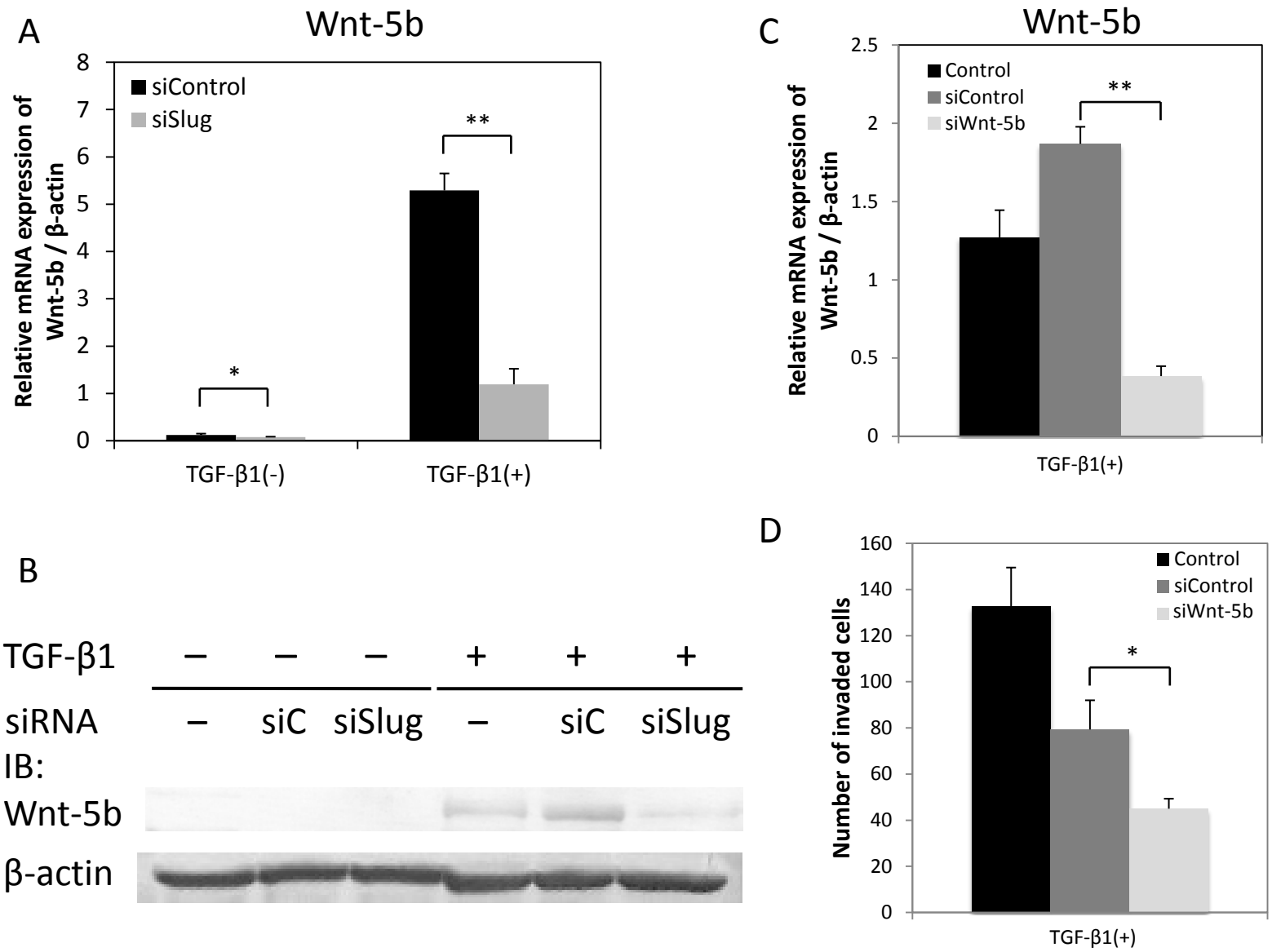
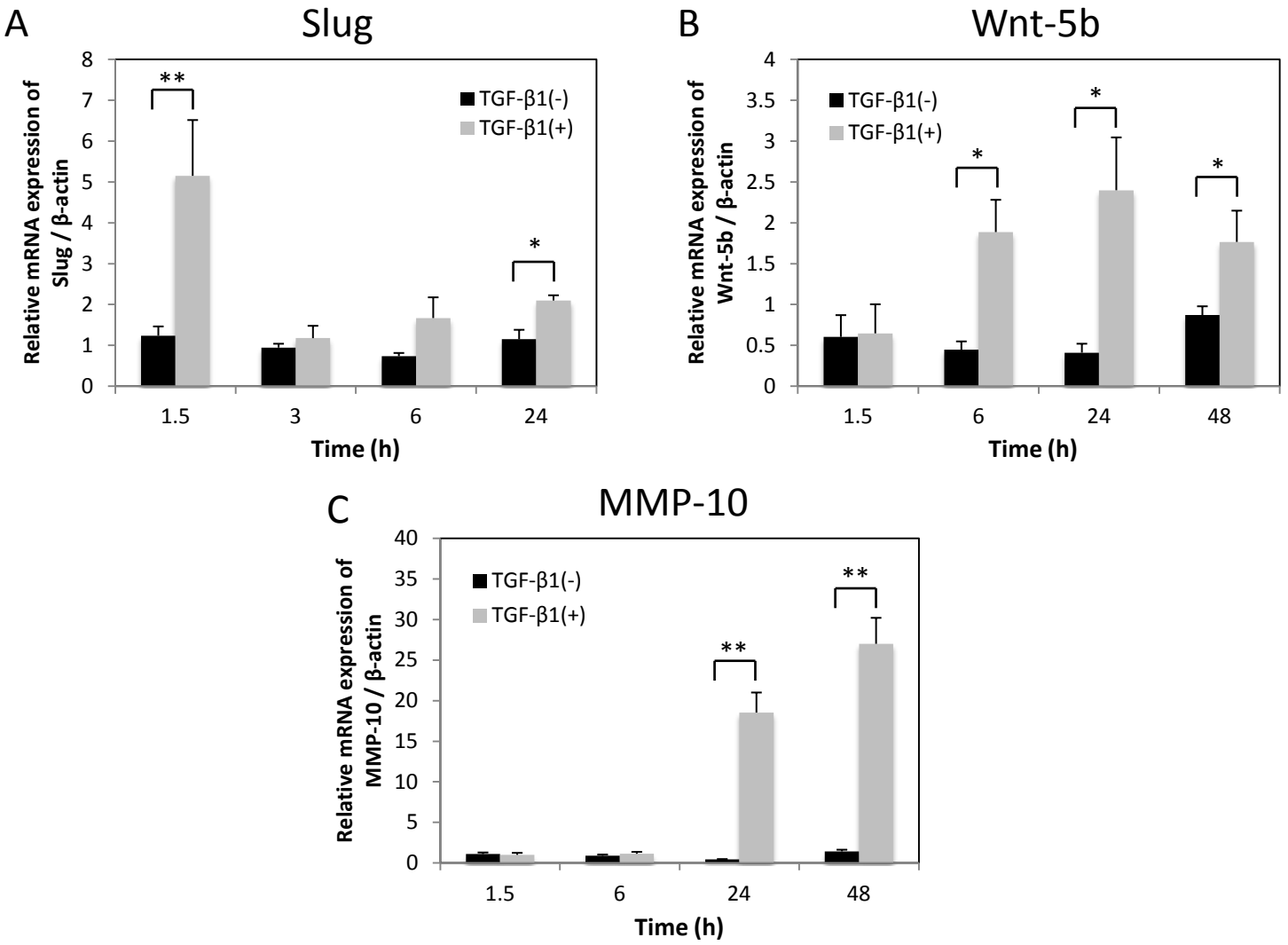

A
Wnt-5b
Relative mRNA expression of Wnt-5b / β-actin
siControl
siSlug
**
*
TGF-β1(-)
TGF-β1(+)
B
TGF-β1
siRNA
IB:
Wnt-5b
β-actin
siC
siSlug
C
Wnt-5b
Control
siControl
siWnt-5b
**
TGF-β1(+)
D
Number of invaded cells
Control
siControl
siWnt-5b
*
TGF-β1(+)

Figure 6.


A
Slug
Relative mRNA expression of Slug / β-actin
TGF-β1(-)
TGF-β1(+)
**
*
0
1
2
3
4
5
6
7
8
1.5
3
6
24
Time (h)
B
Wnt-5b
Relative mRNA expression of Wnt-5b / β-actin
TGF-β1(-)
TGF-β1(+)
*
*
*
0
0.5
1
1.5
2
2.5
3
3.5
4
1.5
6
24
48
Time (h)
C
MMP-10
Relative mRNA expression of MMP-10 / β-actin
TGF-β1(-)
TGF-β1(+)
**
**
0
5
10
15
20
25
30
35
40
1.5
6
24
48
Time (h)