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ORIGINAL ARTICLE

PEG10 counteracts signaling pathways of TGF-β and BMP to regulate growth, motility and invasion of SW1353 chondrosarcoma cells

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Abstract

Recently, we reported highly active transforming growth factor (TGF)-β and bone morphogenetic protein (BMP) signaling in human chondrosarcoma samples and concurrent downregulation of paternally expressed gene 10 (*PEG10*). PEG10 expression was suppressed by TGF-β signaling, and PEG10 interfered with the TGF-β and BMP-SMAD pathways in chondrosarcoma cells. However, the roles of PEG10 in bone tumors, including chondrosarcoma, remain unknown. Here, we report that PEG10 promotes SW1353 chondrosarcoma cell growth by preventing TGF-β1-mediated suppression. In contrast, PEG10 knockdown augments the TGF-β1-induced motility of SW1353 cells. Individually, TGF-β1 and PEG10 siRNA increase AKT phosphorylation, whereas an AKT inhibitor, MK2206, mitigates the effect of PEG10 silencing on cell migration. SW1353 cell invasion was enhanced by BMP-6, which was further increased by PEG10 silencing. The effect of siPEG10 was suppressed by inhibitors of matrix metalloproteinase (MMP). BMP-6 induced expression of MMP-1, -3, and -13, and PEG10 lentivirus or PEG10 siRNA downregulated or further upregulated these MMPs, respectively. PEG10 siRNA increased BMP-6-induced phosphorylation of p38 MAPK and AKT, whereas the p38 inhibitor SB203580 and MK2206 diminished SW1353 cell invasion by PEG10 siRNA. SB203580 and MK2206 impeded the enhancing effect of PEG10 siRNA on the BMP-6-induced expression of MMP-1, -3, and -13. Our findings suggest dual functions for PEG10: accelerating cell growth by suppressing TGF-β signaling and inhibiting cell motility and invasion by interfering with TGF-β and BMP signaling via the AKT and p38 pathways, respectively. Thus, PEG10 might be a molecular target for suppressing the aggressive phenotypes of chondrosarcoma cells.

Keywords PEG10 · Chondrosarcoma · TGF-β · BMP · AKT

Introduction

Paternally expressed gene 10 (*PEG10*) is an imprinted gene, acquired from a retrotransposon to be adapted through mammalian evolution, that plays a crucial role in the formation of the placenta during pregnancy [1]. PEG10 is overexpressed in various cancer types including prostate cancer [2], B cell lymphoma [3, 4], lung cancer [5], gallbladder adenocarcinoma [6], and hepatocellular carcinoma [7–10], playing an important role in the acceleration of cell growth [2, 5, 8]. In addition, PEG10 promotes the epithelial–mesenchymal transition (EMT) in carcinomas to facilitate cell migration and invasion [2, 11–13]. Moreover, the *Peg10* gene is strongly expressed in cartilage primordium of mouse embryos [14]. However, the roles of PEG10 in sarcomas, including bone or cartilage tumors, are unknown.

Yuhei Yahiro and Shingo Maeda contributed equally to this work.

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Transforming growth factor (TGF)-β signaling is a key system in cancers that has dual roles: it inhibits early carcinogenesis by suppressing cell proliferation, whereas during late stages, it promotes tumor progression by accelerating the EMT of carcinomas to drive invasion and metastasis [15]. However, it remains unclear whether the TGF-β family has such dual functions in sarcomas. Members of the TGF-β family, including bone morphogenetic proteins (BMPs), transduce signals through type II and type I receptors by phosphorylation of receptor-regulated Smads (R-Smads). TGF-βs activate SMAD2 or SMAD3, and BMP signaling is mediated by SMAD1, SMAD5, and SMAD9. After forming a trimeric complex with Smad4, activated R-Smads translocate to the nucleus and regulate the transcription of target genes [16]. Interestingly, PEG10 interacts with type I and II TGF-β and BMP receptors to interfere with downstream SMAD signaling in vitro [17]. In addition to canonical SMAD pathways, TGF-β family members transduce signals through non-canonical pathways, such as AKT [18] and p38 mitogen-activated protein kinase (MAPK) [19]. It is not clear whether PEG10 affects these non-canonical pathways or not.

Chondrosarcoma is the second most common primary malignant bone tumor which forms cartilaginous extracellular matrix (ECM), representing 10–20% of malignant bone tumors [20, 21]. Chondrosarcomas seldom respond to chemotherapy or radiotherapy because of the abundance of ECM, the low rate of cell proliferation, and poor vascularity [22–24]. Therefore, wide surgical resection remains the only curative treatment for patients with chondrosarcomas [25]. The TGF-β and BMP-SMAD pathways are active in human chondrosarcoma [26, 27], and exogenous TGF-β1 and BMP-2 increase the migration and invasiveness of chondrosarcoma cells, respectively [28, 29]. Recently, we demonstrated that the expression of PEG10 was increased in human enchondroma, the benign counterpart of chondrosarcomas, whereas phosphorylated (p)-SMAD3 and p-SMAD1/5 accumulated in human high-grade chondrosarcomas, and that PEG10 and p-SMADs were mutually exclusive in clinical specimens of these cartilage tumors [30]. In the chondrosarcoma cell lines SW1353 and Hs 819.T, PEG10 was downregulated and upregulated by exogenous TGF-β1 and BMP-6, respectively, whereas only endogenous TGF-β signaling was responsible for regulating PEG10 levels [30]. Conversely, we confirmed interference of the TGF-β/BMP-SMAD pathway with PEG10 in chondrosarcoma cells; therefore, TGF-β and PEG10 were mutually inhibitory in chondrosarcomas [30]. However, the functions of PEG10 in the growth, migration, and invasion of chondrosarcoma cells have remained elusive.

The aim of this study was to investigate the contribution of PEG10 to the growth, motility, and invasion of SW1353 chondrosarcoma cells in the presence of exogenous TGF-β or BMP. We found the dual roles of PEG10 in the cell growth and motility/invasion of SW1353 cells by interfering with SMADs, p38 MAPK, and AKT in TGF-β and BMP signaling. Our results also show the dual roles of TGF-β in the growth and motility/invasion of chondrosarcoma cells.

Materials and methods

Cell lines and reagents

The human chondrosarcoma cell lines SW1353 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). C28/I2 cells, a cell line established from normal chondrocytes, were a kind gift of Dr. Mary Goldring [31]. The human osteosarcoma cell line Saos-2, the human cervical adenocarcinoma cell line HeLa, the human breast adenocarcinoma cell line MCF-7, and the human hepatocellular carcinoma cell line HepG2 were obtained from ATCC and cultured following provider's culture methods. To stimulate the cells, 1 ng/ml TGF-β1 (PeproTech, Rocky Hill, NJ, USA) or 100 ng/ml BMP-6 (PeproTech) was applied unless otherwise stated. For serum-free cultures, the medium was supplemented with insulin/transferrin/selenium (ITS) (Sigma-Aldrich). SB431542 (Sigma-Aldrich), MK2206 (ChemieTek, Indianapolis, IN, USA), SB203580 (Cell Signaling Technology: CST, Beverly, MA, USA), MMP inhibitor II (Calbiochem, San Diego, CA, USA), and an MMP-13 inhibitor (Calbiochem) were applied at 1 μM, whereas LDN193189 (Sigma-Aldrich) was added at 0.1 μM. Dimethyl sulfoxide (DMSO) was used as the vehicle control at 0.1%. All culture media contained 100 U/ml penicillin G and 100 μg/ml streptomycin.

Expression vectors

The C-terminally V5-tagged mouse *Peg10*-expressing plasmid was described previously [30]. The expression plasmids of constitutively active human ALK2 (ACVR1) and ALK5 (TGFBR1) are a kind gift from Dr. Kohei Miyazono (the University of Tokyo). The lentivirus (LV) expression vector Myc-DDK-tagged lenti ORF clone of human PEG10, transcript variant 1 (# RC229935L1) was purchased from Origene (Rockville, MD, USA). The LV was generated according to the manufacturer's protocol. Experiments using vectors were approved by the Kagoshima University safety control committee for gene-recombination techniques (# 27020). All experiments were performed in accordance with Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of

Living Modified Organisms (Type 2 Use of Living Modified Organisms).

Luciferase assay

Cells were seeded in triplicate in 24-well plates and transfected with 9xCAGA or the BRE luciferase reporter plasmid (a kind gift from Dr. Kohei Miyazono) and the pGL4.75hRlucCMV Renilla vector (Promega, Madison, WI, USA) with or without the expression vectors. Dual luciferase assays were performed as described previously [32] using a GloMax 96 microplate luminometer (Promega).

Immunofluorescence

SW1353 cells were fixed with 4% paraformaldehyde in PBS for 30 min and treated with 0.5% Triton X-100 for 10 min. CAS block (Invitrogen) was used for blocking. Cells were incubated with anti-HA antibody (1:100, 12CA5, Roche, Mannheim, Germany) for 60 min, followed by incubation with anti-mouse IgG Alexa Fluor 568 (1:500, A11061, Thermo Fisher Scientific, Waltham, MA, USA) for 40 min. Subsequently, cells were blocked with CAS block, then incubated with anti-V5-FITC antibody (1:200, R963-25, Invitrogen) for 60 min. Nuclei of cells were stained with Hoechst 33342 (H3570, Thermo Fisher Scientific). Images of immunofluorescence were captured with all-in-one fluorescence microscope BZ-X700/710 (Keyence, Osaka, Japan).

RT-quantitative (q) PCR

Cells were lysed with TRIzol (Invitrogen) to purify RNA, and 1 µg RNA was reverse-transcribed using the Verso cDNA Kit (Thermo Fisher Scientific). The relative expression of gene transcripts was determined by qPCR using SYBR premix Ex Taq II (Takara Bio, Kusatsu, Japan) on a Thermal Cycler Dice TP850 (Takara Bio). PCRs were performed in duplicate per sample, and the level of each gene was normalized to that of *GAPDH*. Experiments were performed in triplicate. Sequence information on the primers is listed in Supplemental Table S1.

siRNA-mediated knockdown of PEG10

Dharmacon ON-TARGETplus SMARTpool for *PEG10* (#L-032579-01; a mixture of four independent siRNAs against human *PEG10*) and the control siRNA pool (#D-001810-10) were purchased from GE Healthcare (Chicago, IL, USA). siRNAs were transfected into cells using Lipofectamine RNAiMax (Invitrogen).

Immunoblotting and ELISA

Cells were lysed in M-PER lysis buffer (Thermo Fisher Scientific) containing aprotinin, sodium orthovanadate, and phenylmethylsulfonyl fluoride and then subjected to SDS–polyacrylamide gel electrophoresis, protein transfer, and chemiluminescence using standard protocols. Blots were incubated with anti-PEG10 (4C10A7, LSBio, Seattle, WA, USA), anti-phospho-AKT (ser-473) (587F11, CST), anti-AKT (5G3, CST), anti-p38 MAPK (D13E1, CST), anti-phospho-p38 MAPK (Thr180/Tyr182) (#9211, CST), anti-Smad1 (#9743, CST), anti-p-Smad1/5/9 (D5B10), CST, anti-Smad2/3 (#610842, BD Biosciences, San Jose, CA, USA), anti-p-Smad3 (C25A9, CST), or anti-tubulin (DM1A, Sigma-Aldrich) and then with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (CST). Chemiluminescent signals were detected on an LAS 4000 Mini Image Analyzer (Fujifilm, Tokyo, Japan). In Fig. 5a, intensities of immunoblot bands of phospho-p38 and p38 obtained from five independent experiments were quantified using ImageJ 1.50i software (National Institutes of Health, USA). The band intensity of phospho-p38 was normalized to that of p38.

The ELISA for MMP-1 was performed using the human MMP1 ELISA Kit (# ab100603, Abcam, Cambridge, UK, USA) according to the manufacturer's instructions. The colorimetric absorbance was measured on a microplate reader (model 1680; Bio-Rad, Hercules, CA, USA).

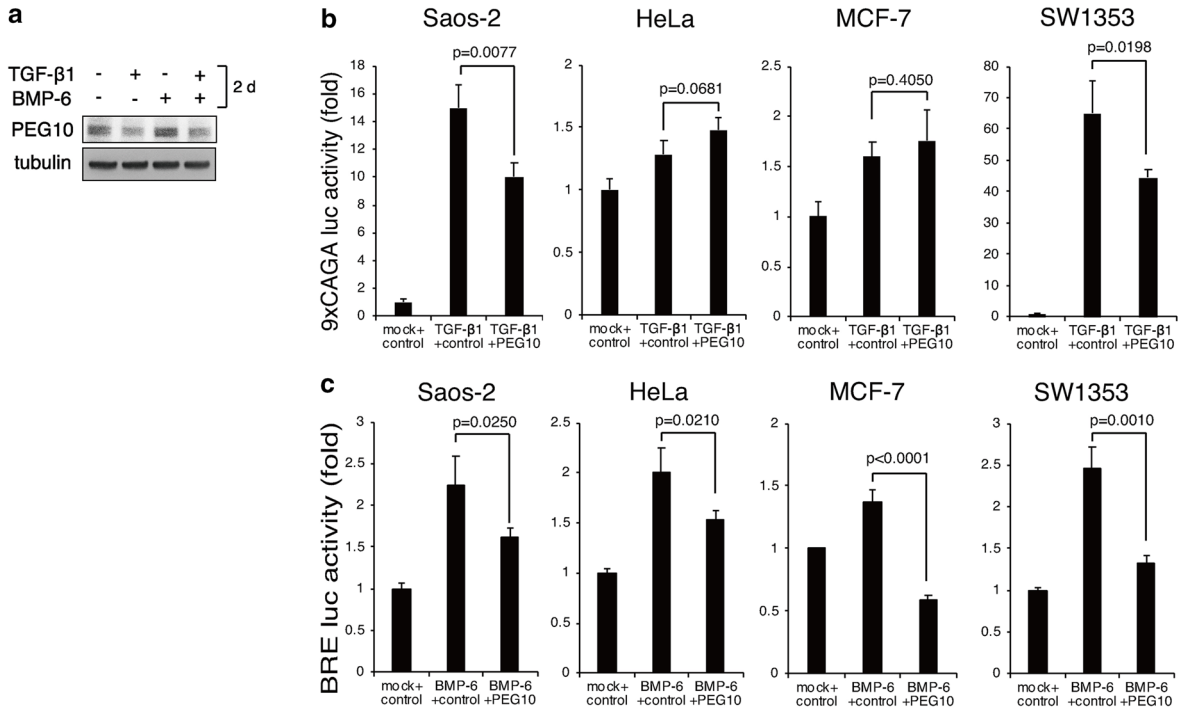
Cell growth analysis

Cells (1000/well) were seeded in 96-well plates ($n = 6$). At the indicated time points, the cells were subjected to water-soluble tetrazolium (WST)-1 cell proliferation assay (Roche) according to the manufacturer's protocol, and the absorbance was measured at 450 nm on a microplate reader.

Cell motility/invasion analyses

Cell motility was assessed by scratch assay ($n = 6$). Prior to scratching, cells were transfected with PEG10 siRNA or control siRNA for 24 h. Using a sterile 200-µl pipette tip, a scratch was made along the monolayer of cells. Microscopic images were recorded just after the scratch and after 12 h to compare the images and calculate the cell migration area using WinROOF image analysis software (Mitani, Tokyo, Japan).

The in vitro invasion assay was performed using a 24-well, 8-µm-pore Matrigel invasion chamber system (#354480, Corning, Corning, NY, USA). Cells transfected with siRNA overnight were pre-treated with inhibitors or 0.1% DMSO as the vehicle control for 2 h. Prior to loading the cells into the upper chamber, the lower chamber was


a
TGF-β1 - + - +
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b
Saos-2
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9xCAGA luc activity (fold)
p=0.0077
p=0.0681
p=0.4050
p=0.0198
mock+ control
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TGF-β1 +PEG10
c
Saos-2
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MCF-7
SW1353
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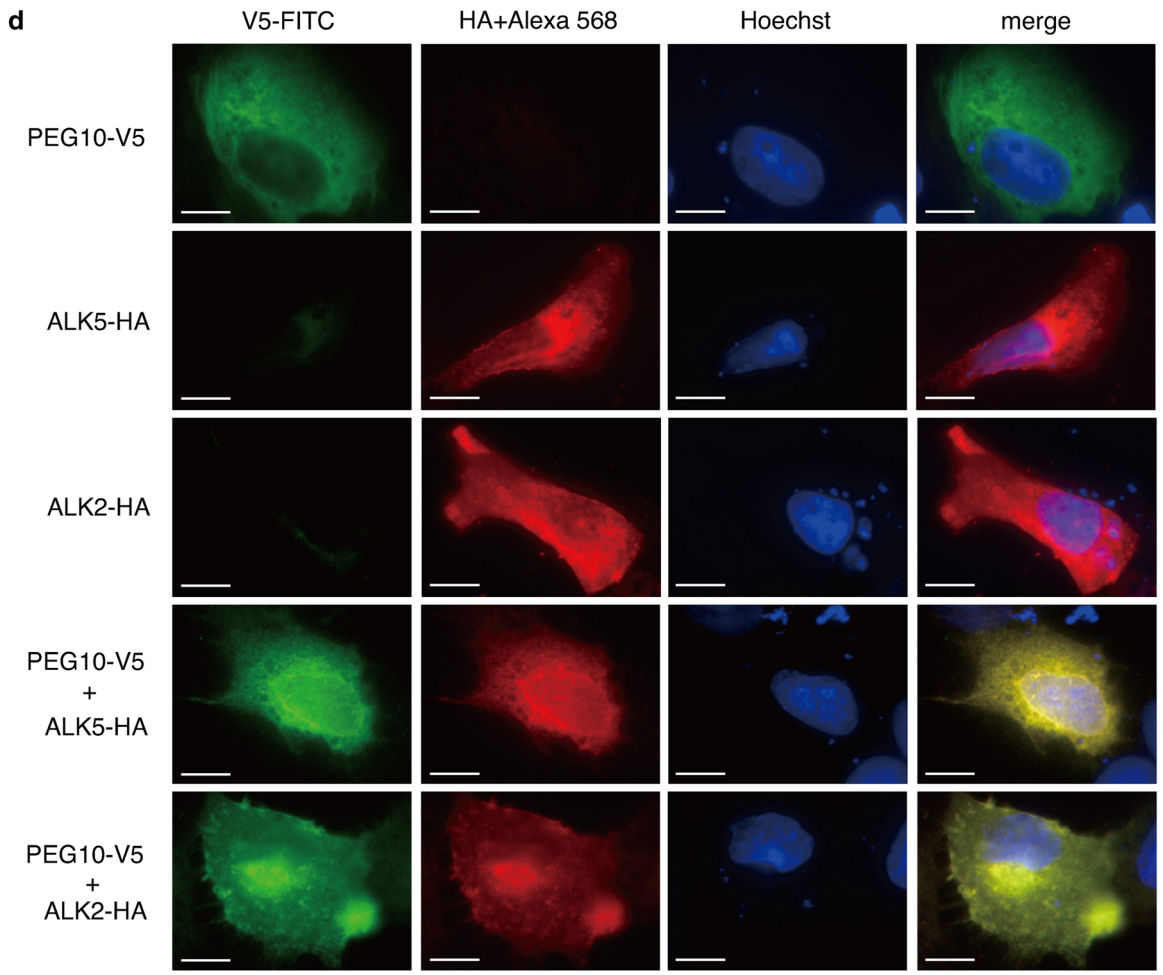
p<0.0001
p=0.0010
mock+ control
BMP-6 +control
BMP-6 +PEG10


d
V5-FITC
HA+Alexa 568
Hoechst
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ALK5-HA
ALK2-HA
PEG10-V5 + ALK5-HA
PEG10-V5 + ALK2-HA

◂**Fig. 1** PEG10 co-localizes with TGF-β and BMP receptors to interfere with the signaling in SW1353 chondrosarcoma cells. **a** Expression of PEG10 in SW1353 chondrosarcoma cells was examined by immunoblotting at 2 days after application of TGF-β1 (1 ng/ml) or BMP-6 (100 ng/ml). Tubulin served as a loading control. **b**, **c** The 9xCAGA or BRE luciferase reporter plasmid together with a *Renilla* reporter were transfected in indicated cells with or without a PEG10 expression vector for overnight, followed by stimulation with TGF-β1 or BMP-6 for 8 h, respectively. Firefly reporter activity was normalized to *Renilla* activity ($n=3$). **d** The V5-tagged PEG10 vector with or without the HA-tagged TGF-β receptor ALK5 or BMP receptor ALK2 plasmids were transfected in SW1353 cells overnight, followed by double immunofluorescence using anti-V5-FITC antibody or anti-HA antibody and Alexa 568-conjugated anti-mouse antibody. Nuclei were visualized with Hoechst dye. Scale bar = 10 μm

filled with medium containing 5% FBS with or without TGF-β1 (1 ng/ml) or BMP-6 (100 ng/ml). Cells were added (2.5×10^5/well) to the serum-free medium in the upper chamber and incubated for 24 h. After the incubation, the cells on the upper surface of the well were removed using a cotton swab. The remaining cells were fixed in methanol for 15 min and then treated with 0.5% Triton X-100 for 5 min and stained with Hoechst dye. The number of cells that had migrated to the lower surface of the filters was counted in three to four fields per filter. Assays were performed in triplicate.

Statistics

Results are expressed as the mean ± 95% confidence interval (CI). Statistical comparisons between various treatments were performed using unpaired Student's *t* test. A value of $p<0.05$ was considered significant.

Results

PEG10 co-localizes with type I receptors of TGF-β or BMP to interfere with the signaling

For stimulating BMP signaling in chondrosarcoma cells, we used BMP-6 rather than other representative BMPs such as BMP-2 because we had observed that expression of BMP-2 and its type I receptors ALK3 (BMPR1A) and ALK6 (BMPR1B) was significantly suppressed in human chondrosarcoma specimens, while levels of BMP-6 and its receptor ALK2 (ACVR1) were not decreased in chondrosarcoma compared to enchondroma samples [30]. We confirmed our recent findings that exogenously applied TGF-β1 or BMP-6 suppresses or increases the expression of PEG10, respectively, in SW1353 chondrosarcoma cells [30] by immunoblotting (Fig. 1a) and RT-qPCR (Fig. 2a). We also confirmed that induction of PEG10 inhibited the activity of TGF-β-SMAD2/3-responsive 9xCAGA luc, as well as BMP-SMAD1/5/9-specific BRE luc, in SW1353 cells [30] (Fig. 1b, c), and checked if this inhibitory actions of PEG10 is universal in other tumor cell types. Indeed, PEG10 plasmid inhibited TGF-β1-activated 9xCAGA reporter in human osteosarcoma cell line Saos-2, however, it failed to affect the activity in human cervical cancer cell line HeLa and in human breast cancer cell line MCF-7 (Fig. 1b). These results raise a possibility that unknown molecule supports the complex formation between PEG10 and TGF-β receptor, which may be absent in HeLa and MCF-7, or inhibitory molecule may exist in these cell lines. In contrast, PEG10 could suppress BMP-6-driven activation of BRE luc in all tested cell lines (Fig. 1c). Next, as Lux et al. reported the co-localization of overexpressed ALK1 or ALK5 and PEG10 in COS-1 cells by immunofluorescence [17], we checked if PEG10 co-localizes with type I receptors of TGF-β1 (ALK5) or BMP-6 (ALK2) in SW1353 chondrosarcoma cells. The co-localizing signals of transfected PEG10 and these receptors were observed in double immunofluorescence in SW1353 cells (Fig. 1d).

PEG10 prevents TGF-β-induced inhibition of growth of SW1353 chondrosarcoma cells

We transfected the pool of four independent PEG10 siRNAs in SW1353 cells to obtain nearly complete silencing of gene expression (Fig. 2a). Because PEG10 is reported to support cell proliferation in hepatocellular carcinomas, whereas knockdown of PEG10 in HepG2 cells inhibits growth [7], we examined the effects of siPEG10 on HepG2 cells as a positive control. WST cell proliferation assays showed a dramatic decline in cell proliferation owing to PEG10 silencing (Fig. S1a). Similarly, the PEG10 siRNA led to substantial suppression of SW1353 cell growth (Fig. 2b), although this effect was weaker than in HepG2 cells, probably because of the extremely high expression of PEG10 in HepG2 cells (about 14-fold higher than in SW1353 cells) (Fig. S1b). Although BMP-6 did not affect the growth rate of SW1353 cells, TGF-β1 suppressed it measurably (Fig. 2c).

TGF-β signaling inhibits the progression of G1 phase of the cell cycle in carcinomas through two mechanisms. First, the TGF-β canonical SMAD2/3 pathway represses expression of the cell cycle driver c-MYC and inhibitors of differentiation (ID) proteins, thereby slowing cell growth [33, 34]. Second, it suppresses cyclin-dependent kinase (CDK) functions by inducing the expression of CDK inhibitors, such as $p15^{Ink4b}$ and $p21^{Waf1}$ [35, 36]. We analyzed the expression levels of these genes in SW1353 and Hs 819.T chondrosarcoma cells by microarray assay, compared with the normal chondrocyte line C28/I2, in combination with TGF-β1 induction. Basic expression of *MYC* was twofold higher in chondrosarcoma cells compared with C28/I2 chondrocytes, but it was not suppressed by TGF-β1 treatment in these cells

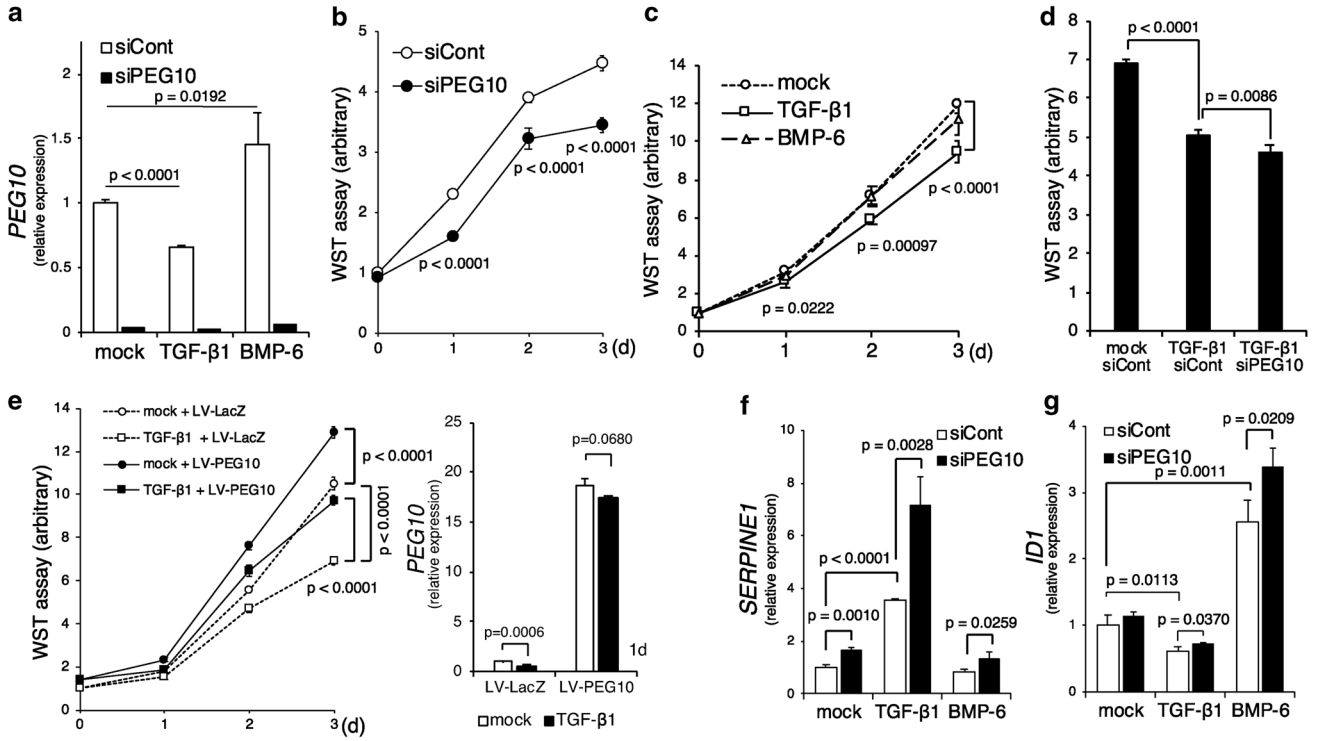
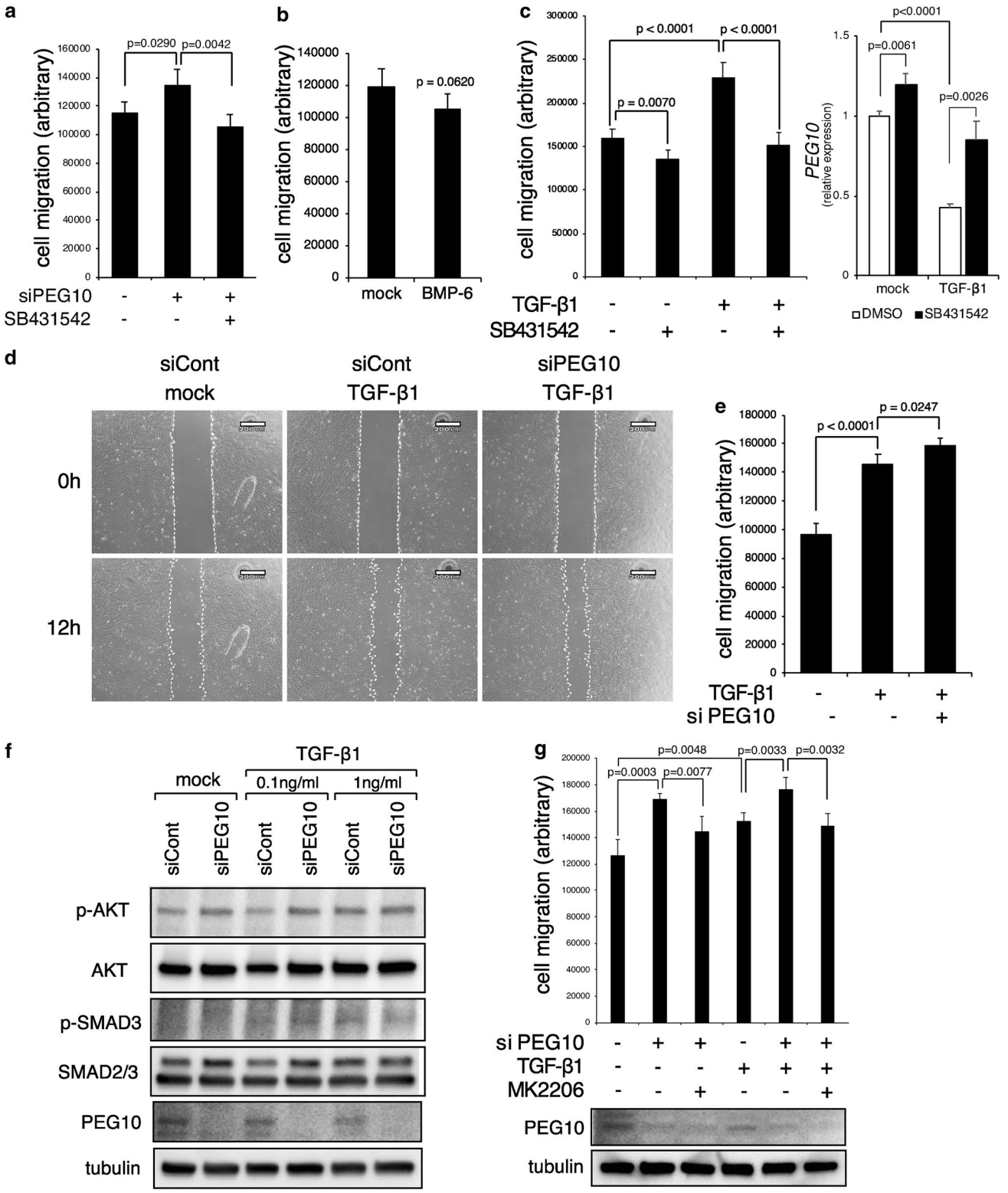


Fig. 2 Mutually contrasting effects of TGF-β1 and PEG10 on the growth of SW1353 chondrosarcoma cells. **a** SW1353 cells were transfected with control (siCont) or PEG10 siRNAs (siPEG10) overnight, followed by treatment with or without TGF-β1 or BMP-6 for 2 days to be subjected to RT-qPCR ($n=3$). **b** SW1353 cells were transfected with control or PEG10 siRNAs overnight, and cell growth was monitored by WST assay daily for 3 days ($n=6$). **c** SW1353 cells were treated with or without TGF-β1 or BMP-6, and cell growth was evaluated by WST assay ($n=6$). **d** SW1353 cells were transfected with control or PEG10 siRNAs overnight, followed by treatment with or without TGF-β1 for 3 days to be subjected to WST assay ($n=6$). **e** SW1353 cells were infected with control LacZ or PEG10-expressing lentivirus (LV) overnight, and then, cell growth was assessed by WST assay ($n=6$). Expression level of PEG10 was monitored in parallel experiments 1 day after TGF-β1 induction by RT-qPCR (right panel, $n=3$). **f**, **g** SW1353 cells were transfected with control or PEG10 siRNA overnight, followed by treatment with or without TGF-β1 or BMP-6 for 2 days to be subjected to RT-qPCR analyses ($n=3$). Results are expressed as the mean ± 95% CI

(Fig. S1c). Indeed, *ID1* was reduced by TGF-β1 in all tested cells (Fig. S1d). Although TGF-β1 showed little effect on the expression levels of *CDKN1A* (p21) and *CDKN1B* (p27) (Fig. S1e, f), it weakly enhanced the expression of *CDKN2B* (p15) and *CDKN1C* (p57) (Fig. S1g, h). Thus, TGF-β signaling appears to inhibit the growth of chondrosarcoma cells, at least in part, by decreasing ID1 expression while mildly increasing p15 and p57 expression. TGF-β1-induced inhibition of SW1353 cell growth was further enhanced by PEG10 silencing, suggesting that the loss of PEG10 enhances TGF-β signaling (Fig. 2d), although the suppressive effect of siPEG10 was mild in the presence of exogenous TGF-β1, probably because the endogenous levels of PEG10 had been already lowered by TGF-β1 treatment.

To further confirm the counteractivity of PEG10 against TGF-β signaling in cell growth, we generated a PEG10-expressing lentivirus (LV) and obtained sufficient overexpression in SW1353 cells (Fig. 2e). LV-PEG10 infection accelerated cell growth and completely rescued

Fig. 3 PEG10 silencing enhances the AKT pathway and TGF-β-induced promotion of SW1353 chondrosarcoma cell migration. **a** SW1353 chondrosarcoma cells were transfected with control or PEG10 siRNAs overnight, followed by treatment with SB431542 (1 μM) or mock control DMSO (0.1%) for 8 h. Cell motility was examined by scratch assay ($n=6$). **b** SW1353 cells were treated with or without BMP-6 (100 ng/ml), followed by scratch assay ($n=6$). **c** SW1353 cells were treated with or without TGF-β1 (1 ng/ml) in combination with SB431542 (1 μM) or mock control DMSO (0.1%) for 24 h to be subjected to scratch assay ($n=6$). Expression level of PEG10 was monitored in parallel experiments by RT-qPCR (right panel, $n=3$). **d**, **e** SW1353 cells were transfected with control or PEG10 siRNA overnight, followed by scratch assay with or without TGF-β1 stimulation ($n=6$). Representative images are shown in **d**. Scale bar = 200 μm. **f** SW1353 cells were transfected with control or PEG10 siRNA overnight, followed by stimulation by TGF-β1 at the indicated concentrations for 20 min and then immunoblotting with the indicated antibodies. **g** SW1353 cells were transfected with control or PEG10 siRNA overnight, pre-treated with MK2206 (1 μM) or 0.1% DMSO for 1 h, and then subjected to scratch assay with or without TGF-β1 in combination with MK2206 or DMSO ($n=6$). Protein level of PEG10 was monitored in parallel experiment by immunoblot. Results are expressed as the mean ± 95% CI



TGF-β1-mediated suppression of growth in SW1353 cells (Fig. 2e). In a recent study, we had found that the loss of PEG10 enhances TGF-β1-induced phosphorylation of SMAD3 and BMP-6-activated p-SMAD1/5/9 [30]. To confirm that PEG10 silencing indeed enhances the TGF-β-SMAD2/3 pathway in SW1353 cells, we measured the expression of *SERPINE1* (PAI1), a representative direct target gene of this pathway, by qRT-PCR. *SERPINE1*

expression was increased by TGF-β1 and enhanced by siPEG10 (Fig. 2f). Similarly, *ID1*, a representative direct target gene of the BMP-SMAD1/5/9 pathway, was upregulated by BMP-6 and further heightened by PEG10 siRNA, although the extent of the enhancement by the loss of PEG10 was less than for *SERPINE1* (Fig. 2g). Thus, these results indicate that the inhibitory role of TGF-β signaling in the growth of chondrosarcoma cells is associated with TGF-β-mediated downregulation of PEG10, which interferes with canonical TGF-β-SMAD2/3 signaling.

PEG10 interferes with the AKT pathway to prevent TGF-β-mediated promotion of SW1353 chondrosarcoma cell motility

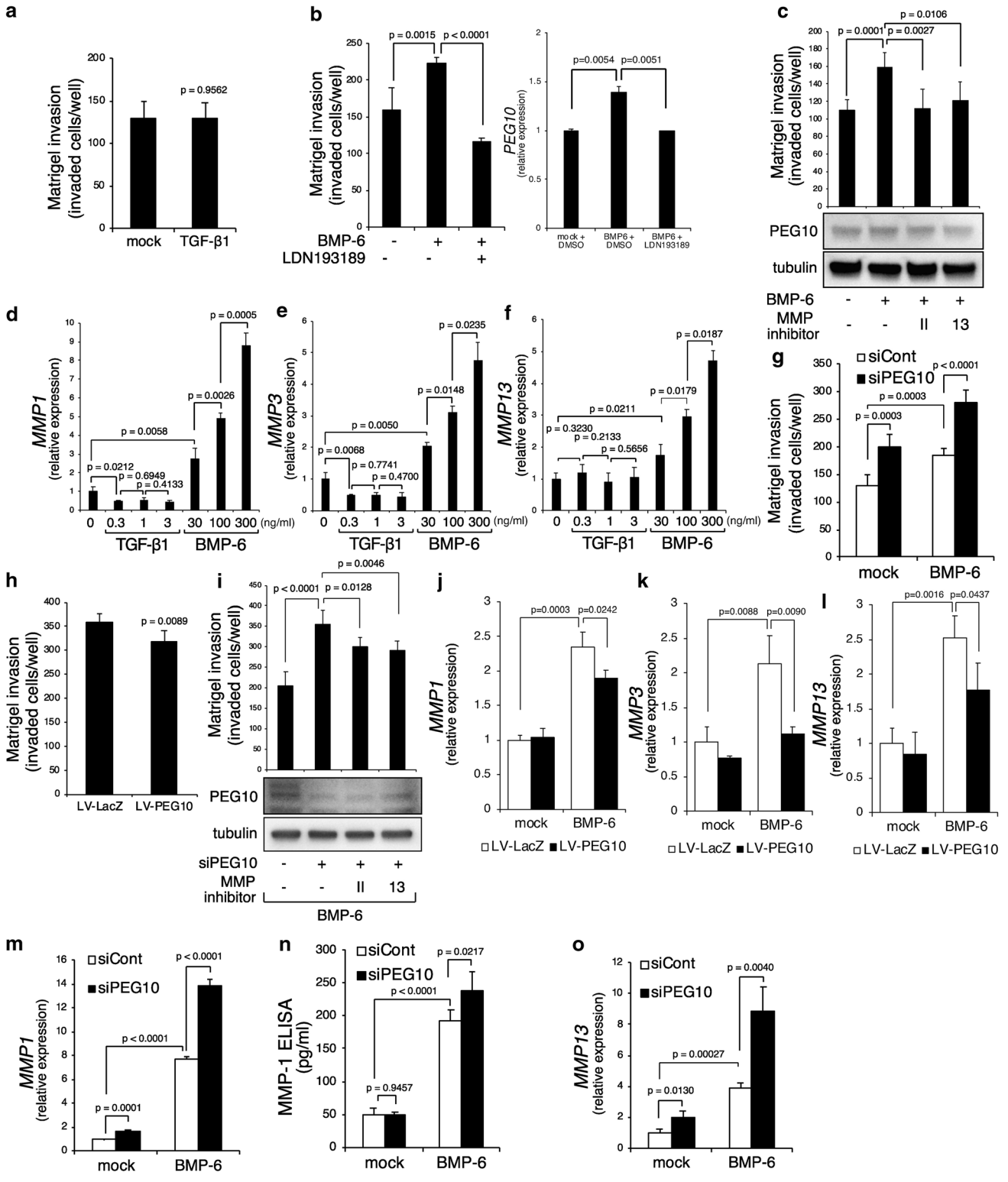
Contrary to reported actions of PEG10 in carcinomas, we found that siRNA-mediated loss of PEG10 increased their motility, as assessed by scratch assay in SW1353 chondrosarcoma cells (Fig. 3a). TGF-β1 increases the motility of the JJ012 human chondrosarcoma cell line through activation of the PI3K/AKT pathway and the promotion of αvβ3 integrin expression [28]. We found that the PEG10 siRNA-mediated promotion of cell migration was blocked by a specific TGF-β receptor inhibitor SB431542 [37] (Fig. 3a), suggesting that PEG10 suppresses cell motility of SW1353 by inhibiting TGF-β signaling. Indeed, although BMP-6 did not affect the migration of SW1353 cells (Fig. 3b), TGF-β1 accelerated their migration, and SB431542 mitigated this effect (Fig. 3c). Importantly, TGF-β1 or SB431542 showed an opposite effect between the expression level of endogenous PEG10 and the rate of cell migration (Fig. 3c). We observed the upregulation of αv integrin (*ITGAV*) and β3 integrin (*ITGB3*) by TGF-β1 induction in SW1353 and Hs 819.T chondrosarcoma cells by microarray assay (Fig. S2a, b), suggesting that upregulation of αvβ3 integrin was responsible for the promoting effect of TGF-β signaling in SW1353 cell motility. siPEG10 mildly enhanced the promoting effects of TGF-β1 in cell migration (Fig. 3d, e). This weak effect of siPEG10 in the presence of TGF-β1 was similar to that in Fig. 2d, which might have been due to the lower PEG10 expression that was induced by TGF-β1. In SW1353 cells, knockdown of PEG10 increased the phosphorylation of AKT, suggesting AKT pathway to be involved in the effect of siPEG10 in SW1353 cell motility (Fig. 3f). At 20 min after TGF-β1 application, siPEG10 slightly enhanced TGF-β1-induced SMAD3 phosphorylation, although we detected an increase in p-SMAD3 by PEG10 silencing 60 min after TGF-β1 induction [30], raising the possibility that the binding of PEG10 to TGF-β receptors to block SMAD activation depends on the time after ligand induction and inhibition of AKT activity by PEG10 starts earlier.

Upon blocking the AKT pathway with MK2206, an allosteric oral AKT1/2/3 inhibitor being tested in a number of phase 2 clinical trials against advanced solid tumors [38], the promoting effect of siPEG10 was eliminated, indicating that the loss of PEG10 strengthens the TGF-β-AKT axis (Fig. 3g). We confirmed that MK2206 did not alter protein level of PEG10 (Fig. 3g). Because MK2206 counteracted siPEG10 with regard to SW1353 cell motility, we asked if the TGF-β-AKT pathway contributes to cell growth. However, MK2206 did not block the suppressive effects of TGF-β1 by WST assay in SW1353 cells (Fig. S2c), suggesting that the canonical SMAD2/3 pathway is the main mediator in cell growth.

Fig. 4 Expression of MMPs and matrix gel invasion of SW1353 cells are increased by BMP-6 stimulation and inhibited by PEG10. **a** SW1353 cells were subjected to Matrigel invasion assay with or without TGF-β1 (1 ng/ml) ($n=11$). **b**, **c** SW1353 cells were subjected to Matrigel invasion assay with or without BMP-6 (100 ng/ml) in combination with DMSO (0.1%), LDN193189 (0.1 μM) (**b**, $n=9$), MMP inhibitor II (1 μM), or an MMP-13 inhibitor (1 μM) (**c**, $n=11$). Expression level of PEG10 was monitored in parallel experiments by RT-qPCR (**b**, right panel, $n=3$) or immunoblot (**c**). **d**–**f** SW1353 cells were treated with or without TGF-β1 or BMP-6 at the indicated concentrations for 2 days to be subjected to RT-qPCR analysis ($n=3$). **g** SW1353 cells were transfected with control or PEG10 siRNA overnight, followed by Matrigel invasion assay with or without BMP-6 ($n=9$). **h** SW1353 cells were infected with control LacZ- or PEG10-expressing lentivirus overnight and then subjected to Matrigel invasion assay ($n=11$). **i** SW1353 cells were transfected with control or PEG10 siRNA overnight, followed by Matrigel invasion assay with BMP-6 in combination with DMSO, MMP inhibitor II (1 μM), or the MMP-13 inhibitor (1 μM) ($n=11$). Protein level of PEG10 was monitored in parallel experiment by immunoblot. **j**–**l** SW1353 cells were infected with control or PEG10-expressing LV overnight, followed by stimulation with mock or BMP-6 for 2 days to be subjected to RT-qPCR analysis ($n=3$). **m**–**o** SW1353 cells were transfected with control or PEG10 siRNA overnight, followed by treatment with or without BMP-6 for 2 days. Cell lysates were subjected to RT-qPCR analysis of *MMP1* (**m**) and *MMP13* (**o**) ($n=3$). Conditioned medium was examined for levels of secreted MMP-1 by ELISA (**n**) ($n=3$). Results are expressed as the mean ± 95% CI

PEG10 prevents BMP-mediated promotion of matrix metalloproteinase expression and invasion of SW1353 chondrosarcoma cells

High-grade chondrosarcoma, in which TGF-β/BMP signaling is highly active, is aggressive, invades, and metastasizes. These malignant processes require cells to produce proteases, such as matrix metalloproteinases (MMPs), to degrade the surrounding ECM and basement membrane [39]. To investigate the roles of TGF-β/BMP signaling and PEG10 in chondrosarcoma cell invasion, we employed Matrigel invasion chambers. In SW1353 cells, TGF-β1 had no effect on cell invasion (Fig. 4a). In contrast, application of BMP-6 accelerated cell invasion substantially, which was blocked by LDN193189, a specific inhibitor of type I BMP receptors, that impede the SMAD and non-SMAD pathways of p38 and AKT [40], indicating that the action



was type I BMP receptor-specific (Fig. 4b). We confirmed PEG10 to be increased by exogenous BMP-6 and decreased by LDN193189 (Fig. 4b, right panel).

We had evaluated the expression levels of all MMP genes in C28/I2, SW1353, and Hs 819.T cells by microarray analysis. Compared with C28/I2 normal chondrocytes, chondrosarcoma cells upregulated *MMP1*, *MMP2*, *MMP3*, *MMP14*, *MMP19*, and *MMP13* (Fig. S3a). Accordingly, we assessed the contribution of MMPs to BMP-stimulated cell invasion using MMP inhibitor II, which is specific for

MMP-1, -3, -7, and -9 [41], and an MMP-13 inhibitor [42] and found that both MMP inhibitors completely abrogated the effect of BMP-6 (Fig. 4c), while they showed no effect on PEG10 protein expression, suggesting that the production of MMPs by BMP-6 signaling is responsible for the enhanced cell invasion. Among the substantially expressed MMP genes in chondrosarcoma cells (Fig. S3a), MMP-1 (collagenase 1), MMP-3 (stromelysin 1), and MMP-13 (collagenase 3) promote the motility and invasiveness of chondrosarcoma cells [29, 43, 44]. Although TGF-β1 failed to increase the expression of *MMP1*, *MMP3*, and *MMP13*, BMP-6 promoted their expression in a dose-dependent manner (Fig. 4d–f). siRNA-mediated knockdown of PEG10 significantly increased the rate of SW1353 cell invasion, regardless of BMP-6 induction (Fig. 4g). Conversely, forced expression of PEG10 by LV transduction mildly but significantly suppressed cell invasion (Fig. 4h). The endogenous level of PEG10 may be sufficient to suppress BMP signaling, therefore additional exogenous LV-PEG10 showed mild effects. In the presence of BMP-6, siPEG10-mediated enhancement of cell invasion was partially reversed by MMP inhibitors (Fig. 4i). Again, MMP inhibitors did not alter PEG10 expression level. BMP-6-induced expression of *MMP1*, *MMP3*, and *MMP13* was eliminated by PEG10 LV infection (Fig. 4j–l). Conversely, PEG10 knockdown significantly increased BMP-6-mediated induction of *MMP1* expression (Fig. 4m). Essentially the same result was achieved at the protein level by MMP-1 ELISA using conditioned media from SW1353 cell cultures (Fig. 4n). Similar results were obtained regarding the expression of *MMP13* (Fig. 4o). These results suggest that PEG10 suppresses expression of MMPs via interfering BMP signaling to inhibit MMPs-mediated cell invasion. *MMP2* (encoding gelatinase A) was the most abundant MMP gene in SW1353 and Hs 819.T cells (Fig. S3a), but the promoting effect of BMP-6 and siPEG10 was weak (Fig. S3b). Among the MMPs that accumulated in chondrosarcoma cells, only *MMP14* encodes a transmembrane type MMP that is approximately 5- to 7-fold less efficient than MMP-1 in collagen digestion [45]; therefore, we excluded MMP14 from our study.

PEG10 suppresses p38 and AKT pathways to regulate expression of MMPs and invasion of SW1353 chondrosarcoma cells

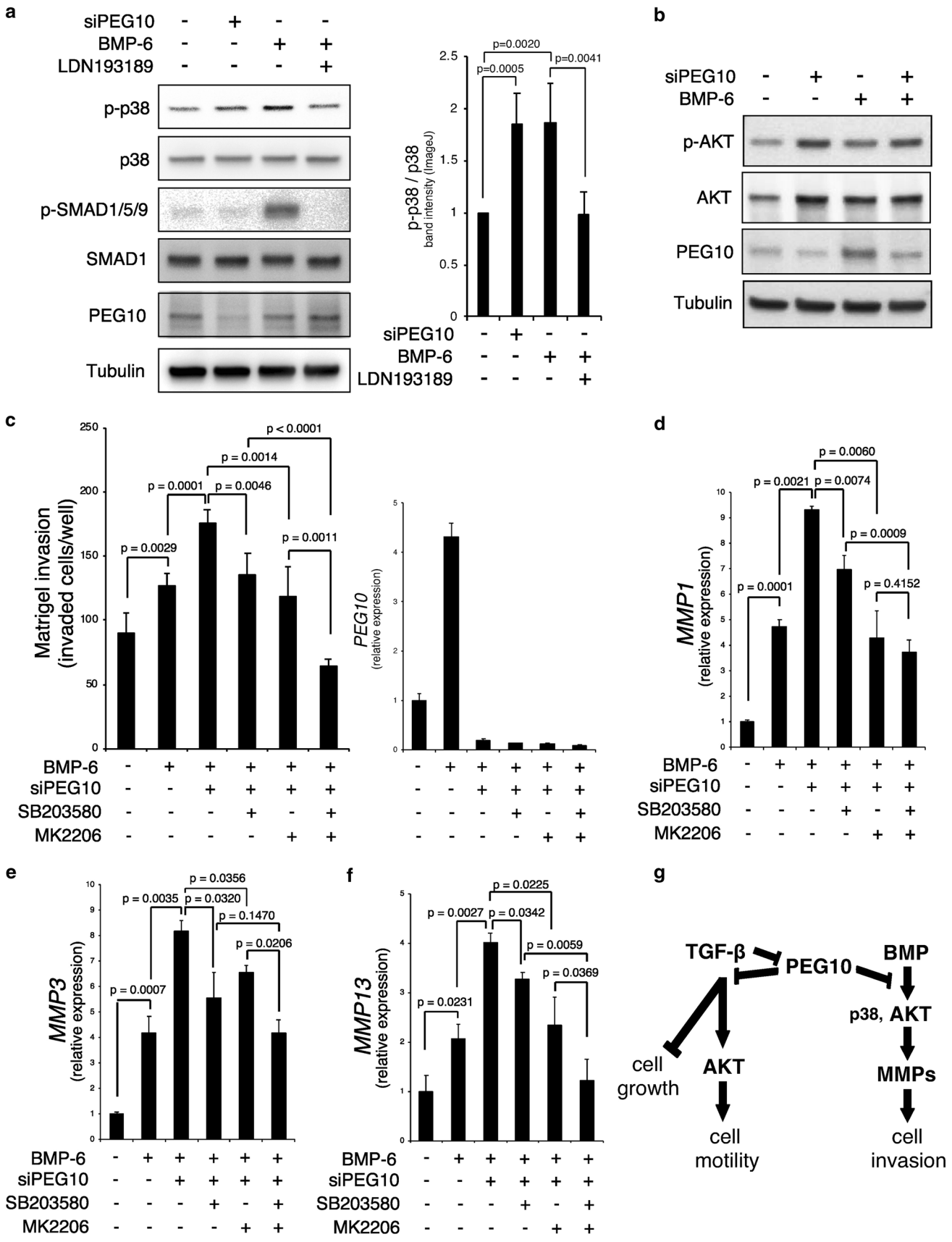
The p38 MAPK pathway upregulates *MMP1* and *MMP3* by stabilizing their mRNAs [46], whereas LDN193189 inhibits BMP-mediated activation of p38, and AKT, in addition to SMAD1/5/9 [40]. Accordingly, we examined whether p38 MAPK affects the expression of *MMPs* in SW1353 chondrosarcoma cells. Phosphorylation level of p38 was almost doubled by BMP-6 to be abolished by LDN193189, while PEG10 knockdown also increased the phosphorylation

Fig. 5 Inhibitor of p38 MAPK or AKT counteracts the promoting effects of PEG10 siRNA on BMP-6-induced MMP expression and invasion of SW1353 cells. **a** SW1353 chondrosarcoma cells were transfected with control or PEG10 siRNA overnight, pretreated with DMSO (0.1%) or LDN193189 (0.1 μM), treated with BMP-6 (100 ng/ml) for 1 h, and then subjected to immunoblotting with the indicated antibodies. The band intensity of p-p38 and p38 was quantitated using ImageJ software, and the amount of p-p38 was normalized to that of p38 (right panel, $n=5$). **b** SW1353 cells were transfected with control or PEG10 siRNA overnight, followed by treatment with or without BMP-6 for 10 min, and then subjected to immunoblotting with the indicated antibodies. **c** SW1353 cells were transfected with control or PEG10 siRNA overnight and then subjected to Matrigel invasion assay with or without BMP-6 in combination with DMSO (0.1%), SB203580 (1 μM), and/or MK2206 (1 μM) ($n=8$). Expression level of PEG10 was monitored in parallel experiments by RT-qPCR (right panel, $n=3$). **d–f** SW1353 cells were transfected with control or PEG10 siRNA overnight, followed by treatment with or without BMP-6 in combination with DMSO, SB203580, and/or MK2206 for 2 days to be subjected to RT-qPCR analysis ($n=3$). Results are expressed as the mean ± 95% CI. **g** Diagram illustrating the results obtained from experiments with SW1353 chondrosarcoma cells. (1) TGF-β signaling plays dual roles in chondrosarcoma cells: inhibition of cell growth and promotion of cell migration via the AKT pathway. (2) BMP signaling promotes cell invasion via p38 and AKT pathways. (3) PEG10 plays dual roles by counteracting these effects of TGF-β and BMP, while expression of PEG10 is suppressed by TGF-β

similarly as BMP-6 (Fig. 5a). The PI3K/AKT pathway is activated by BMP-2 [47] and promotes MMP-13 production [48] in JJ012 human chondrosarcoma cells. In SW1353 cells, our immunoblotting results showed that BMP-6 increased the phosphorylation of AKT at 10 min, while siPEG10 enhanced it (Fig. 5b). SB203580, a p38 MAPK-specific inhibitor [49], and MK2206 completely abolished the siPEG10-mediated increase in cell invasion, whereas the combined administration of SB203580 and MK2206 had additive suppressive effects, while these inhibitors had no effect against PEG10 expression level (Fig. 5c). The elevated expression of *MMP1, MMP3,* and *MMP13* by BMP-6 and siPEG10 was substantially suppressed by SB203580 and MK2206, although the additive effect of their combination was seen only with regard to *MMP3* and *MMP13* expression (Fig. 5d–f). These results suggest that the p38 and AKT pathways are important for BMP signaling in promoting MMPs expression and subsequent cell invasion to be blocked by PEG10.

Discussion

The results obtained from experiments with SW1353 chondrosarcoma cells in this study are illustrated in Fig. 5g. First, TGF-β signaling plays dual roles: inhibition of cell growth and promotion of cell migration via the AKT pathway. Second, BMP signaling promotes cell invasion via p38 and AKT pathways. Finally, PEG10 counteracts these


a
siPEG10 - + - -
BMP-6 - - + +
LDN193189 - - - +
p-p38
p38
p-SMAD1/5/9
SMAD1
PEG10
Tubulin
p-p38 / p38
band intensity (ImageJ)
p=0.0020
p=0.0005
p=0.0041
b
siPEG10 - + - +
BMP-6 - - + +
p-AKT
AKT
PEG10
Tubulin
c
Matrigel invasion
(invaded cells/well)
p = 0.0029
p = 0.0001
p = 0.0046
p = 0.0014
p < 0.0001
p = 0.0011
PEG10
(relative expression)
BMP-6
siPEG10
SB203580
MK2206
d
MMP1
(relative expression)
p = 0.0001
p = 0.0021
p = 0.0074
p = 0.0060
p = 0.0009
p = 0.4152
e
MMP3
(relative expression)
p = 0.0007
p = 0.0035
p = 0.0320
p = 0.0356
p = 0.1470
p = 0.0206
f
MMP13
(relative expression)
p = 0.0231
p = 0.0027
p = 0.0342
p = 0.0225
p = 0.0059
p = 0.0369
g
TGF-β
PEG10
BMP
p38, AKT
MMPs
cell invasion
AKT
cell motility
cell growth

effects of TGF-β and BMP signaling, while expression of PEG10 is suppressed by TGF-β signaling. Thus, the dual roles of TGF-β signaling in SW1353 chondrosarcoma cells are in close association with suppression of PEG10 expression, that PEG10 plays contrary biphasic roles: promotion of cell growth and preventing cell motility and invasion. Interestingly, these actions of PEG10 are contrary to the well-known functions of TGF-β in cancer, that TGF-β signaling inhibits early carcinogenesis by suppressing cell proliferation, whereas it accelerates the EMT of carcinomas to drive invasion and metastasis during late stages [15]. In addition to SMADs, ERK, RhoA, p38 MAPK, and PI3K/AKT have been implicated in TGF-β-induced EMT [15, 50–52]. Although TGF-β increases the motility of JJ012 chondrosarcoma cells [28, 29, 47], its potential dual function in chondrosarcoma cells has remained undefined. In this study, we found that TGF-β1 and BMP-6 promoted the migration and invasion of SW1353 chondrosarcoma cells, respectively, consistent with previous reports [28, 29, 47] (Figs. 3, 4, 5). The actions of TGF-β1 and BMP-6 were elicited through the AKT and p38 MAPK pathways and accompanied by increased MMPs expression, which also agrees with previous studies in JJ012 cells [28, 29, 47, 53]. Importantly, we found that the silencing of PEG10 significantly enhances cell motility and invasion and MMP expression. Lux et al. [17] reported that ALK1, a type I receptor of TGF-β and BMP-9, formed a complex with PEG10 via C-terminal ALK1/PEG10-RF1 interaction domains (APID-1/2). The interaction was not exclusive for ALK1, that other ALKs including ALK5 and ALK2 were also immunoprecipitated with PEG10, while co-localization of ALK1 or ALK5 with PEG10 was detected by immunofluorescence. Similarly, we recently reported [30] and show in this study that PEG10 co-localized with ALK5 or ALK2 to suppress TGF-β or BMP signaling. Because both APID-1 and -2 reside in the kinase domain of ALK1, binding of PEG10 may change conformation of the kinase domain to interfere with phosphorylation (activation) of not only SMADs, but also AKT and p38 mediated by ALKs. Indeed, upon PEG10 silencing, phosphorylation level of p38 and AKT (Fig. 5) as well as SMADs [30] was increased. To our knowledge, this is the first study to show the role of PEG10 to affect phosphorylation level of AKT and p38. These evidences indicate that the functions of PEG10 in the phenotypes of SW1353 chondrosarcoma cells are mediated, at least in part, by interference of signaling through TGF-β and BMP receptors. Therefore, our results have led us to speculate that in higher grade chondrosarcomas with suppressed PEG10 expression, cells become resistant to PEG10-mediated inhibition of the TGF-β-AKT, BMP-AKT, and -p38 MAPK axes and gain the ability to migrate and metastasize. In enchondromas with high PEG10 expression, PEG10 eliminates TGF-β/BMP signaling to prevent malignant tumor progression, whereas cell growth is accelerated by PEG10 because of the lack of TGF-β signaling-mediated inhibition.

This study has several weaknesses. We could not conduct in vivo animal experiments, because a xenograft animal model for chondrosarcoma for studying invasion and metastasis has not been established. In addition, although the expression profiling assays were performed in SW1353 and Hs 819.T chondrosarcoma cells, our present results were generated using SW1353 chondrosarcoma cells. However, our results on the association of TGF-β/BMP and AKT/p38 MAPK on cell migration/invasion are similar to those in studies using JJ012 human chondrosarcoma cells [28, 29, 47, 53]; therefore, SW1353 cells are a representative line of human chondrosarcoma cells. Using these cell line systems, we need to determine the effects of molecular targets in regulating malignant progression of chondrosarcomas, which seldom respond to chemotherapy or radiotherapy [22–24]. Inhibiting TGF-β or BMP signaling using neutralizing antibodies or inhibitor compounds against type I receptors, the AKT or p38 MAPK pathways may regulate the progression of chondrosarcomas; however, because these signaling pathways mediate a broad range of processes, the side effects will be severe. Therefore, PEG10 might be a more specific molecular target that can control the progression of chondrosarcoma.

In conclusion, we demonstrated that PEG10 counteracts the effects of TGF-β on cell growth inhibition and the promotion of cell motility, whereas it suppresses BMP-induced cell invasion of SW1353 chondrosarcoma cells. These actions of PEG10 in cell migration and invasion are mediated through blockade of the TGF-β-AKT and BMP-p38/BMP-AKT signaling axes.

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Compliance with ethical standards

Statement of human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest All authors declare that they have no conflicts of interest regarding the contents of this article.

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