Abstract. Squamous cell carcinoma is the most common cancer in the oral cavity. We previously demonstrated that transforming growth factor-β1 (TGF-β1) promotes the epithelial-mesenchymal transition (EMT) of human oral squamous cell carcinoma (hOSCC) cells; however, it remains to be clarified whether the TGF-β superfamily member bone morphogenetic protein (BMP) affects this process in hOSCC cells. Here, we examined the independent and collective effects of TGF-β1 and BMP-2 on EMT and mesenchymal-epithelial transition (MET) in a panel of four hOSCC cell lines. Notably, we found that HSC-4 cells were the most responsive to BMP-2 stimulation, which resulted in the upregulation of Smad1/5/9 target genes such as the MET inducers ID1 and cytokeratin 9 (CK9). Furthermore, BMP-2 downregulated the mesenchymal marker N-cadherin and the EMT inducer Snail, but upregulated epithelial CK9 expression, indicating that BMP-2 prefers to induce MET rather than EMT. Moreover, TGF-β1 dampened BMP-2-induced epithelial gene expression by inhibiting Smad1/5/9 expression and phosphorylation. Functional analysis revealed that TGF-β1 and BMP-2 significantly enhanced HSC-4 cell migration and proliferation, respectively. Collectively, these data suggest that TGF-β positively regulates hOSCC invasion in the primary tumor, whereas BMP-2 facilitates cancer cell colonization at secondary metastatic sites. Thus, the invasive and metastatic characteristics of hOSCC appear to be reciprocally regulated by BMP and TGF-β.

Introduction

Epithelial-mesenchymal transition (EMT) is characterized by the loss of epithelial markers with a reciprocal gain in mesenchymal phenotype and migratory potential (1). While EMT is essential for embryonic development and adult tissue maintenance (2,3), it is also necessary for desmoplasia and cancer cell migration (4). Conversely, mesenchymal-epithelial transition (MET) is a physiological and embryological phenomenon induced by cytokines (5,6). During EMT, transcription factors, such as Snail (7) and Slug (8) are upregulated and induce mesenchymal gene expression and suppress that of epithelial genes (9).

Transforming growth factor-β1 (TGF-β1) is an important inducer of EMT (10,11). Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily, of which 20 have been discovered in humans to date (12). BMPs were first identified for their pro-osteogenic effects, but recent studies have revealed their additional significance as tissue morphogenetic factors (13), particularly for BMP-2, -4 and -7 (14). In particular, BMP-2 is a cytokine used to treat bone defects and is being investigated in regenerative studies (15,16).

BMP signaling is induced when a heterodimeric membrane kinase binds BMP and subsequently triggers Smad protein phosphorylation, similar to the mechanism of TGF-β pathway activation. However, downstream BMP-induced signals are mediated by Smad1/5/9, whereas TGF-β signaling is mediated by Smad2/3. These receptor-regulated Smad complexes
(Smad1/5/9 and Smad2/3) bind the common mediator Smad4, and collectively translocate into the nucleus. The Smad complex then binds the DNA promoter region upstream of target genes to induce their expression (17). The effects of BMP on cancer cells are relatively uncharacterized when compared to those of TGF-β, but generally thought to promote EMT (18,19). However, BMP-2 was recently reported to suppress EMT in the presence of TGF-β (20). Thus, the overall effects of BMP on EMT likely depend on the cellular context.

Squamous cell carcinoma is the most common cancer of the oral cavity in humans (21,22). Although BMPs are thought to be involved in cancer metastasis, the mechanisms underlying BMP-induced EMT/MET have not yet been clarified at the molecular level (12). Nevertheless, a few studies have demonstrated the positive effects of BMP on cancer progression using human oral squamous cell carcinoma (hOSCC) cell lines and carcinoma tissue (23-26). Moreover, while bone is a major target for hOSCC metastasis (26,27), the cellular and molecular mechanisms facilitating this process remain to be elucidated.

Previous studies by our group revealed that TGF-β1 responsiveness was correlated with EMT-related gene expression in six hOSCC cell lines. Notably, TGF-β1 enhanced the migration of HSC-4 hOSCC cells via the Slug/Wnt-5b/MMP-10 and integrin α3β1/FAK signaling axes (28,29). Since cells are subjected to multiple simultaneous signals from extracellular ligands and must then integrate and interpret them, this study investigated the independent and collective effects of TGF-β1 and BMP-2 on EMT and MET in HSC-4 cells. In addition, we evaluated how TGF-β1 affects the BMP-2-induced MET in HSC-4 cells at the molecular level.

Materials and methods

Materials. Cell lines were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). Recombinant human TGF-β1 and BMP-2 were purchased from Peprotech, Inc. (Rocky Hill, NJ, USA). The TGF-β1 receptor kinase inhibitor SB431542 was provided by Merck-Millipore (Frankfurt, Germany). The BMP type 1 receptor kinase inhibitor LDN-193189 was purchased from Selleck Chemical (Houston, TX, USA). The proteasome inhibitor MG132 was obtained from Merck Millipore. The protease inhibitor cocktail and phosphatase inhibitor cocktail 1 and 2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All of the other reagents were of analytical grade.

Cell culture. All of the cell lines were grown at 37°C in 5% CO₂. Human HSC-2 and HSC-4 squamous cell carcinoma cells were cultured in MEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). SAS cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% FBS. HSC-3 cells were cultured in DMEM (Sigma-Aldrich) containing 10% FBS. The culture medium was replaced with serum-free medium 24 h prior to cytokine-stimulated experiments. In the majority of experiments, 2.0x10⁵ hOSCC cells were cultured in 500 µl serum-free media containing 10 ng/ml TGF-β1 or 20 ng/ml BMP-2 for the indicated time-periods in 24-well tissue culture plates.

Western blot analysis. For Smad phosphorylation analysis, 3.0x10⁴ cells were lysed in RIPA buffer (Sigma-Aldrich) containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich) and the protein content was assessed with a BCA reagent (Thermo Fisher Scientific, Waltham, MA, USA). To examine marker protein expression, 1.0x10⁶ cells were cultured in a 6-well plate in serum-free MEM with or without 10 ng/ml TGF-β1 for the indicated time-points. Harvested cells were homogenized in SDS sample buffer containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich). Proteins were separated by 12.5% SDS-PAGE (ATTO Co., Tokyo, Japan) and subsequently transferred onto PVDF membranes (Merck Millipore). The membranes were probed with the primary antibodies, including rabbit anti-Smad1 (#6944) and rabbit anti-Smad5 (#9517; both from Cell Signaling Technology, Beverly, MA, USA), rabbit anti-Smad9 [Smad8 (R-64); Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA] and rabbit anti-phospho-Smad1 (Ser463/465)/Smad5 (Ser463/465)/Smad9 (Ser465/467) (#13820; Cell Signaling Technology). Mouse anti-β-actin (clone C4; Santa Cruz Biotechnology, Inc.) was used as a loading control in the Smad phosphorylation experiments. The blots were then incubated with alkaline phosphatase-conjugated secondary antibody and signals were visualized using an alkaline phosphatase substrate kit (BCIP/NBT substrate kit; Vector Laboratories, Inc., Burlingame, CA, USA).

Reverse transcriptase quantitative-PCR (RT-qPCR). For total RNA preparation, 2.0x10⁵ cells were cultured in 24-well tissue culture plates. Total RNA was isolated using the Isogen reagent (Nippon Gene, Co., Ltd., Toyama, Japan) according to the manufacturer's instructions. RNA was reverse transcribed into first-strand cDNA with an RT-PCR system kit (Takara Bio, Inc., Shiga, Japan). qPCR was performed on a Thermal Cycler Dice Real-Time System (Takara Bio, Inc.) using SYBR® Premix Ex Taq™ II (Takara Bio, Inc.) with human gene-specific primers (Table I). Target gene expression was normalized to an internal β-actin reference and expressed in terms of fold-change relative to the control sample (30).

Proliferation assay. HSC-4 cell proliferation was evaluated by alamarBlue® assay (AbD Serotec, Oxon, UK) according to the manufacturer's instructions. Briefly, 1.0x10⁵ cells/well were subcultured in 96-well plates in MEM supplemented with 10% FBS for 24 h. The culture medium was then replaced with MEM supplemented without FBS with or without TGF-β1 (10 ng/ml) or BMP-2 (20 ng/ml) for 2 days. The cells were washed once with PBS and then incubated with 100 µl alamarBlue® (10% alamarBlue® in Ham's F-12) at 37°C in 5% CO₂ for 1.5 h. The absorbances at 570 and 600 nm were used to measure the reduced and oxidized forms of reagent, respectively, using a microplate reader. Assays were performed independently at least 3 times (n=6).

Cell migration assays. Boyden chamber migration assays were performed using cells transfected withSlug siRNA as aforementioned. Then, cells were treated with 10 ng/ml TGF-β1 or 20 ng/ml BMP-2 under serum-free conditions for 72 h and subsequently plated at a density of 1.0x10⁵ cells in the upper chamber of a Boyden apparatus in serum-free media,
and were allowed to migrate into the lower chamber containing medium supplemented with 10% FBS for 24 h at 37°C. The chamber filter was fixed in 4% paraformaldehyde and stained with DAPI (1:500; Sigma-Aldrich) for 10 min. Migrating cells were counted in 9 fields on the membrane. Values indicate the mean number of migrating cells compared to the controls.

Statistical analysis. All experiments were performed at least 3 times independently. Results are expressed as the means ± standard deviation (SD). Data were analyzed using two-tailed, independent Student's t-tests. P<0.05 was considered statistically significant.

Table I. Primer sequences for the qPCR analysis.

| Genes      | Primer sequences                |
|------------|---------------------------------|
| BMP-2      | F 5'-AAGATTCCTAAGGATGCCTGTCGCTGTC-3' |
|            | R 5'-TCGTCAGTGCAGCTGTCGTCGTCGTCGTC-3' |
| Cytokeratin 9 | F 5'-TCAGCTGACTGGCAGAACAAC-3' |
|            | R 5'-ACCCTCACGCTGACTGCTGCTGCTGCTGTC-3' |
| Cytokeratin 18 | F 5'-AGGAGTATAGGGCCCCCGCTGCTGAA-3' |
|            | R 5'-TTGGCTTAGGTTGCTGCTGTCCTGTCGTC-3' |
| E-cadherin | F 5'-TACACTGCAGCAGGACAGAAA-3' |
|            | R 5'-TTGGCAACAGTGTCCGAGAT-3' |
| ID1        | F 5'-CGGAATCTGAGAAGGAAACAG-3' |
|            | R 5'-CTGAGAAGAACACAAAAGCTGAG-3' |
| N-cadherin | F 5'-CAATGGATGAAAGACGACCCATC-3' |
|            | R 5'-TCGTCAGTGCAGCTGTCGTCGTCGTC-3' |
| NEDD4      | F 5'-GATTTGTAACCGGAACCTGCAAGA-3' |
|            | R 5'-CCAGTCCATTGACAACATCCTGTCGTC-3' |
| NEDD4L     | F 5'-CCAATGGTCAGAAGTAATGTTCGTCGTC-3' |
|            | R 5'-AAGGCATTACCTGCTGCTGCTGCTGTC-3' |
| Smad1      | F 5'-ACAGCTGCTGAGAAGGAAACAG-3' |
|            | R 5'-TGAGTTCAGAAGGAAACAG-3' |
| Smad5      | F 5'-GCTTTATACCCACACACTGCTGTCGTC-3' |
|            | R 5'-CCTGCGGTAGATATCTGTC-3' |
| Smad6      | F 5'-GAGATCTGAGAAGGAAACAG-3' |
|            | R 5'-AGATGCATTGAGGAGGAGGT-3' |
| Smad7      | F 5'-TCGACACCCATCACCATTGCTGTC-3' |
|            | R 5'-TCGTCAGTGCAGCAGCAG-3' |
| Smad9      | F 5'-TGCCCAAGTCAATCTGACGAGGAG-3' |
|            | R 5'-CATGAGTAGATGAACTTCAATCCGACA-3' |
| Smurf1     | F 5'-CCTGTCAGAAGGAGGAGGAGGT-3' |
|            | R 5'-CCTGTCAGAAGGAGGAGGAGGT-3' |
| Smurf2     | F 5'-TGCAAGTTAACTTCAATCCGACA-3' |
|            | R 5'-CTTTCAGTCCACACAGAATCCGACA-3' |
| Snail      | F 5'-GACCAATATGCCGAGGAGGT-3' |
|            | R 5'-TCGCTGCTGCTGCTGCTGCTGCTGTC-3' |
| β-actin    | F 5'-GGAGATTATGCTGCTGCTGCTGCTGCTGCTGTC-3' |
|            | R 5'-GACTCATTGAGGCTGCTGCTGCTGCTGTC-3' |

F, forward; R, reverse.

Figure 1. Difference in BMP-2 responsiveness in the hOSCC cell lines. Expression of BMP-2 target genes Smad6, Smad7 and ID1 was evaluated in (A) HSC-4, (B) HSC-3, (C) HSC-2 and (D) SAS hOSCC cell lines following treatment with 10 ng/ml BMP-2 for 3 h (light gray bars) vs. untreated controls (dark gray bars). Data represent the mean ± SD from triplicate experiments (**P<0.01). hOSCC, human oral squamous cell carcinoma; BMP, bone morphogenetic protein.

Results

BMP-2 responsiveness in the hOSCC cell lines. We first studied the cellular response to BMP-2 in four hOSCC cell lines: HSC-2, HSC-3, HSC-4, and SAS. Smad6, Smad7 and ID1 are targets of BMP signaling (31). Expression analysis revealed that BMP-2 induced a significant upregulation of all three target genes in the HSC-4 cells (Fig. 1A), but no marked changes were observed in the other cell lines (Fig. 1B-D). Thus, we used HSC-4 cells to investigate the effects of
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BMP-2 on MET in the following experiments. In addition, HSC-4 cells are also responsive to TGF-β1 stimulation based on our previous examinations (28).

**BMP-2-induced response in HSC-4 cells.** To confirm BMP-2 signaling in HSC-4 cells, the time course of Smad6 and ID1 gene expression was investigated in the presence or absence of LDN-193189, a selective inhibitor of activin receptor-like kinase (ALK)-2/3. The results showed that both genes were upregulated 1 h after BMP-2 treatment, peaking at 3 h post-stimulation, which was significantly inhibited by LDN-193189. Moreover, Smad1/5/9 phosphorylation was increased in the BMP-2-treated HSC-4 cells, but inhibited in the presence of LDN-193189 (Fig. 2C). Thus, these results indicated that BMP-2 elicits Smad1/5/9 activation and target gene expression in HSC-4 cells.

**Effects of BMP-2 or TGF-β1 on epithelial and mesenchymal status in HSC-4 cells.** Next, we examined how BMP-2 alters the epithelial and mesenchymal characteristics of HSC-4 cells by examining marker expression with RT-qPCR (Fig. 3). Notably, the epithelial marker cytokeratin 9 (CK9) was clearly upregulated following BMP-2 stimulation (Fig. 3A, right), whereas that of N-cadherin was significantly suppressed (Fig. 3C, left). Conversely, TGF-β1 stimulation resulted in the significant suppression of epithelial cytokeratin 18 (CK18) marker (Fig. 3B, right), as well as the induction of the mesenchymal markers N-cadherin and vimentin (Fig. 3D). However, the expression of epithelial marker E-cadherin was not affected by either BMP-2 or TGF-β1 stimulation (Fig. 3A and B, left).
and mesenchymal vimentin was not significantly affected by BMP-2 (Fig. 3C, right). Moreover, the morphology of BMP-2-treated HSC-4 cells was not different from that of control cells (data not shown). Collectively, these data indicated that TGF-β1 suppresses epithelial marker expression and promotes that of mesenchymal markers, resulting in EMT. In contrast, BMP-2 has the opposite effect promoting MET.

**TGF-β1 abrogates BMP-2-mediated effects on MET/EMT gene expression in a dose-dependent manner.** To examine the function of TGF-β1 on BMP-2-induced MET, HSC-4 cells were treated with increasing doses of TGF-β1 with or without BMP-2 stimulation. BMP-2-induced epithelial CK9 (Fig. 4A, left) and ID1 upregulation (Fig. 2B), whereas the N-cadherin and Snail downregulation (Fig. 4A, right) was significantly suppressed by LDN-193189. Moreover, BMP-2-induced CK9 and ID1 upregulation was significantly suppressed by TGF-β1 in a dose-dependent manner (Fig. 4B and C). Similarly, BMP-2-induced N-cadherin and Snail downregulation were significantly suppressed by TGF-β1 stimulation in a dose-dependent manner (Fig. 4D and E).

**Effect of TGF-β1 on Smad1/5/9 expression and phosphorylation.** Western blot analysis showed that TGF-β1 hindered Smad1/5/9 phosphorylation in HSC-4 cells in the presence or absence of BMP-2 (Fig. 5A). In addition, while TGF-β1 suppressed Smad1 expression independently of BMP-2, it had no marked effect on Smad5 expression. Smad9 expression was undetectable regardless of exogenous stimulation. With respect to RNA expression, both Smad1 and 9 were significantly decreased by TGF-β1 stimulation, whereas that of Smad5 was unaffected (Fig. 5B). TGF-β1 stimulation alters the levels of phosphorylated (p)Smad1/5 and pSmad2 by regulating the E3 ligase activities of Smurf1 (32,33), NEDD4 (34) Smurf2 (35) and NEDD4L (36), respectively. Notably, Smurf1 and NEDD4 were significantly upregulated by TGF-β1 stimulation, whereas Smurf2 and NEDD4L were mostly unchanged (Fig. 5C). However, the TGF-β1-mediated degradation of BMP-2-induced pSmad1/5/9 remained intact after treatment with MG132 proteasome inhibitor. This suggested that BMP signal attenuation by TGF-β1 occurs in a proteasome-independent manner (Fig. 5D), possibly through the Smad1/9 downregulation (Fig. 5B). Thus, the effect of
TGF-β1 on BMP-2 signaling likely results from the suppression of Smad1/9 expression rather than phosphorylation.

Effect of BMP-2 and TGF-β1 on HSC-4 cell migration and proliferation. The effect of BMP-2 on cell migration and proliferation was investigated to assess its potential effect on hOSCC progression (Fig. 6). This analysis revealed that TGF-β1 significantly enhanced the migratory capacity of HSC-4 cells 72 h after TGF-β1 stimulation, whereas BMP-2 did not (Fig. 6A). Alternatively, BMP-2 facilitated cell proliferation 48 h after stimulation, but was significantly suppressed in the presence of TGF-β1 (Fig. 6B).

Discussion

In order to detect BMP-2-responsive hOSCC, Smad6 and ID1 expression (Fig. 1) and Smad1/5/9 phosphorylation (Fig. 2) were examined in four hOSCC cell lines after BMP-2 stimulation. HSC-4 cells were the most responsive to both BMP-2 and TGF-β1 and thus selected for further analysis. Results from the present study demonstrate that BMP-2 promoted the expression of epithelial CK9, but suppressed that of the mesenchymal markers N-cadherin and vimentin (Fig. 3C), suggesting that it promotes MET. Moreover, these BMP-2-inducible effects were significantly inhibited by TGF-β1 stimulation in HSC-4 cells.
in a dose-dependent manner (Fig. 4B-E). Collectively, these results strongly suggest that TGF-β1 inhibits BMP-2-induced MET in hOSCC.

Further analysis revealed that TGF-β1 suppressed BMP-2-induced Smad1/5/9 phosphorylation (Fig. 5A) and Smad1/9 expression (Fig. 5B) and promoted the expression of E3 protein ligases that target TGF-β2 pathway effectors in HSC-4 cells (Fig. 5C). As such, TGF-β1 is likely a key regulator of Smad1/5/9 pathway suppression downstream of BMP-2 in hOSCC cells. ID (inhibitor of DNA binding) proteins are a family of four transcriptional regulators, including ID1 (31). ID1 expression is reportedly enhanced by BMP-2-induced Smad1/5/9 signaling (Fig. 5a) and migration (Fig. 6a) and invasion (29) in primary hOSCC cells. In addition, TGF-β1-induced EMT in a dose-dependent manner (Fig 4E), suggesting that TGF-β1 suppresses renal interstitial fibrosis by regulating epithelial-mesenchymal transition. Biochim Biophys Acta 1840: 2621-2634, 2014.

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