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A dual role of TGF-β in human osteoclast differentiation mediated by Smad1 versus Smad3 signaling

Bitnara Lee[a,b], Younseo Oh[a,c], Sungsin Jo[a], Tae-Hwan Kim[a,**], Jong Dae Ji[c,*]

[a] Hanyang University Hospital for Rheumatic Diseases, Seoul, South Korea
[b] Research Institute of Molecular Medicine and Nutrition, Korea University, Seoul, South Korea
[c] Rheumatology, College of Medicine, Korea University, Seoul, South Korea

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ABSTRACT

TGF-β1 is highly expressed in the synovial tissue of patients with rheumatoid arthritis and is known as a cytokine that plays an important role in tissue repair and immune cell regulation. However, the role of TGF-β1 is still unclear in osteoclastogenesis. In this study, we examined the effect of TGF-β1 on osteoclast differentiation and the underlying mechanism using healthy human peripheral blood monocytes. TGF-β1 was found to inhibit osteoclast differentiation and decrease the expression of osteoclast-specific genes such as acid phosphatase 5, tartrate resistant and cathepsin K. Levels of NFAT1, an important transcription factor in osteoclastogenesis, were also reduced. In addition, TGF-β1 suppressed receptor activator of NF-κB (RANK) ligand-induced NF-κB and p38 MAPK signaling. Inhibition of osteoclast differentiation by TGF-β1 was reversed by 1 μM SB431542 (an inhibitor of ALK4/5/7), which inhibited TGF-β1-induced phosphorylation of SMAD1, but not that of SMAD3. TGF-β1 also restricted RANK expression, and this was partially reversed by 1 μM SB431542. In contrast, the inhibition of SMAD3 by SIS3 (an inhibitor of SMAD3) reduced the osteoclast formation. TGF-β1 has both inhibitory and stimulatory effects on human osteoclast differentiation, and that these opposing functions are mediated by SMAD1 and SMAD3 signaling, respectively.

1. Introduction

Osteoclasts are multinucleated giant cells derived from cells of the mononuclear hematopoietic myeloid lineage, and are involved in inflammatory bone destruction such as that observed in rheumatoid arthritis. Many cytokines and growth factors are implicated in osteoclast differentiation and functional regulation [1]. TGF-β1 is one of the most abundant cytokines in the bone matrix, into which it is released excessively due to increased osteoclastic bone resorption associated with rheumatoid arthritis [2–4].

Previous studies have reported different effects of TGF-β1 on osteoclasts, depending on the culture system used [5,6]. TGF-β1 has been shown to directly stimulate osteoclastogenesis by increasing receptor activator of NF-κB (RANK) expression in RAW 264.7 murine monocytes [7,8]. In contrast, it has been reported to inhibit osteoclast formation in co-culture with osteoblasts by decreasing RANK ligand (RANKL) expression [8]. Although a similar co-culture system has been used with murine cells, different results have been obtained according to the TGF-β dose administered. Low TGF-β concentrations stimulate osteoclast differentiation by affecting the RANKL/osteoprotegerin ratio, whereas high concentrations suppress such differentiation [9]. Recent studies have reported that TGF-β plays a central role in murine osteoclastogenesis through the binding of SMAD3 to the TRAF6-TAB1-TAK1 complex and reciprocal cooperation between SMAD2/3 and c-Fos [10,11]. In addition, when TGF-β signals are blocked using 1 μM SB431542, a specific inhibitor of TGF-β type I receptor kinase activity, osteoclast differentiation is largely suppressed [10]. These results show that TGF-induced SMAD3 signaling may be important in the promotion of murine osteoclast differentiation. In human osteoclastogenesis, previous studies have demonstrated that continuous exposure to TGF-β abrogates osteoclast formation through down-regulation of RANK expression [12] and is also involved in inhibiting the activity of osteoclasts through induction of their apoptosis [13].

TGF-β signaling is stimulated via heterotetrameric complexes of type I and type II dual-specificity kinase receptors. Activated receptors of TGF-β induce SMAD signaling, as well as non-SMAD signaling such as that involving ERK1/2, JNK, and p38 MAPK. TGF-β generally induces phosphorylation of SMAD2 and 3, whereas bone morphogenetic

* Corresponding author at: Rheumatology, College of Medicine, Korea University, 126-1, Anam-Dong 5-Ga, Sungbuk-Ku, Seoul, 136-705, South Korea.
** Corresponding author at: Rheumatology, Hanyang University Hospital for Rheumatic Diseases, 222-1, Wangsimni-ro, Seongdong-gu, Seoul, 04763, South Korea.
E-mail addresses: thkim@hanyang.ac.kr (T.-H. Kim), jjdjmesy@korea.ac.kr (J.D. Ji).

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proteins typically phosphorylate SMAD1, 5, and 8. Activated receptor-regulated SMADs then form trimeric complexes with the common mediator SMAD4 that are translocated to the nucleus, where they act as transcription factors [14]. Further studies, however, have revealed that TGF-β activates not only canonical SMAD2/3-mediated signaling, but also SMAD1/5-mediated signaling, which is more typical of bone morphogenetic protein stimulation [15]. For example, in endothelial cells, in addition to canonical TGF-β receptor II/ALK5-mediated SMAD2/3 phosphorylation, SMAD1/5 phosphorylation can be induced by TGF-β receptor II following its formation of a complex with the ALK1/ALK5 heterodimer upon binding of TGF-β. This process depends on the kinase activity of ALK5 and the accessory receptor endoglin. It has also been reported that TGF-β1 activates the SMAD1/5 pathway in mature human macrophages by complexing with the ALK1/ALK5 heterodimer [16]. These results indicate that TGF-β may have contrasting functions in the same cells, according to the balance between SMAD1/5- and SMAD2/3-dependent signaling. The present study demonstrates that osteoclast differentiation is affected differently by TGF, depending on whether SMAD3 or SMAD1 signal transduction is induced.

2. Materials and methods

2.1. Reagents

Recombinant human TGF-β1 was obtained from R&D Systems (Minneapolis, MN, USA). M-CSF and soluble RANKL were purchased from PeproTech (Rocky Hill, NJ, USA). SB431542 (an inhibitor of ALK4/5/7) and SIS3 (an SMAD3 inhibitor) were supplied by MedChemExpress (Monmouth Junction, NJ, USA). Small interfering RNA (siRNA) targeting human *SMAD1* (cat. no. L-012723-00) and corresponding control siRNA (cat. no. D-001510-10) were purchased from Dharmacon (Lafayette, CO, USA).

2.2. Cell culture

PBMCs were isolated from the blood of a healthy donor by density gradient centrifugation with Ficoll (Invitrogen, Carlsbad, CA, USA). Monocytes were then isolated from the PBMCs using anti-CD14 magnetic beads (Miltenyi Biotec, Auburn, CA, USA). To obtain osteoclast precursors, monocytes were exposed to 20 ng/ml human M-CSF in α-MEM (Invitrogen) with 10% FBS for 2 days. This study was approved by the Institutional Review Board (IRB 2008-09-001) of Hanyang University Hospital for Rheumatic Disease.

2.3. Osteoclast differentiation

Monocytes were cultured in the presence of 20 ng/ml M-CSF for 2 days to generate osteoclast precursors, which were subsequently incubated with 20 ng/ml M-CSF and 40 ng/ml RANKL for an additional 6 days. The culture medium was changed every 3 days. On day 8, the cells were fixed and stained for acid phosphatase 5, tartrate resistant (ACP5, also known as TRAP) using an acid phosphatase leukocyte diagnostic kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's recommendations. Multinucleated (with more than three nuclei) ACP5-positive osteoclasts were counted in triplicate wells.

2.4. Bone resorption assay

Monocytes were seeded into the wells of a calcium phosphate-coated plate (Biocoat Osteologic 16-well Multitest Slide, BD Biosciences, Baltimore, MD, USA) and cultured for up to 8 days under osteoclastogenic culture conditions. The cells were removed by 5% sodium hypochlorite (Sigma-Aldrich) treatment in order to observe resorption pits under a light microscope.

2.5. Western immunoblotting

Cell lysates were separated by SDS-PAGE on 10% gels and transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Amersham, UK). The membranes were incubated with antibodies against specific proteins, and the resulting bands were visualized using ECL Western Blotting Substrate (Thermo Fisher Scientific, Foster City, CA, USA). These proteins included phospho-SMAD3, SMAD3, phospho-SMAD1, SMAD1, endoglin, phospho-p38, p38, phospho-ERK, ERK, NF-κB p65 (Cell Signaling, Danvers, MA, USA), lamin B, RANK (Santa Cruz Biotechnology, Dallas, TX, USA), matrix metallopeptidase 9 (MMP9) (R &D Systems), NFAT1 (BD Biosciences), and β-actin (Sigma-Aldrich).

2.6. RNA interference

Monocytes were cultured with 40 ng/ml M-CSF for 5 days, before being transfected using Lipofectamine RNAiMAX transfection reagent (Invitrogen) for 2 days with 100 nM *SMAD1* siRNA or control siRNA in the presence 20 ng/ml M-CSF. Some of the cells were then stimulated for 1 day with 10 ng/ml TGF-β.

2.7. Quantitative reverse transcription PCR (qRT-PCR)

RNA was isolated using TRIzol reagent (Invitrogen/Life Technologies, Carlsbad, CA, USA), and 0.5 μg total RNA was reverse transcribed using a First Strand cDNA Synthesis kit (Fermentas, Hanover, MD, USA). qPCR was performed using an iCycler iQ™ thermal cycler and detection system (Bio-Rad Laboratories, Hercules, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad) following the manufacturer's protocols. Expression of target genes was normalized to that of *GAPDH*. The primers used for qPCR were as follows: *SMAD1*: 5′-ACCTGCTTAC CTGCCTCCTG-3′ and 5′-CATAAGCAACCGCCTGAACA-3′; *SMAD3*: 5′-GCCTGTGCTGGAACATCATC-3′ and 5′-TTGCCCTCATGTGTGCT CTT-3′; endoglin (*ENG*): 5′-CCACTGCACTTGGCCTACA-3′ and 5′-GCC CACTCAAGGATCTGG-3′; *ACP5*: 5′-TGGCTTTGCCTATGTGGA-3′ and 5′-CCTGGTCTTAAAGAGGGACTT-3′; cathepsin K (*CTSK*): 5′-CTCTTCC ATTTCTTCCACGAT-3′ and 5′-ACACCAACTCCCTTCCAAAG-3′; RANK (*TNFRSF11A*): 5′-CCATCATCTTTGGCGTTTG-3′ and 5′-AGCTGTGAGT GCTTTCCCT-3′; *MMP9*: 5′-GTCACCTATGACATCCTGCAGTG-3′ and 5′-CTTTCCTCCAGAACAGAATACCAGTT-3′; and *GAPDH*: 5′-ATCAAGA AGGTGGTGAAGCA-3′ and 5′-GTCGCTGTTGAAGTCAGAGGA-3′.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Results are expressed as means ± SD. The Wilcoxon signed-rank test was applied to evaluate group differences, with *p*-values less than 0.05 being considered significant.

3. Results

3.1. Expression of SMAD1 and endoglin is induced during human osteoclast differentiation

To assess the expression of SMAD1 and SMAD3 during human osteoclast differentiation, we analyzed their mRNA and protein levels. Expression of SMAD3 decreased, whereas that of SMAD1 increased in the early stage of differentiation before decreasing in the late stage (Fig. 1A, B). Endoglin is a co-receptor of TGF-β that acts as such in SMAD1 signaling. As SMAD1 expression was induced during osteoclastogenesis, we also examined levels of endoglin during this process. Expression of endoglin increased together with that of SMAD1 (Fig. 1C, D), and was further enhanced on day 8 by TGF-β1 (Fig. 2A, B). We next used a recombinant human M-CSF Receptor/Fc chimera (M-CSF R/Fc) to specifically block M-CSF signaling and confirm that the increase in

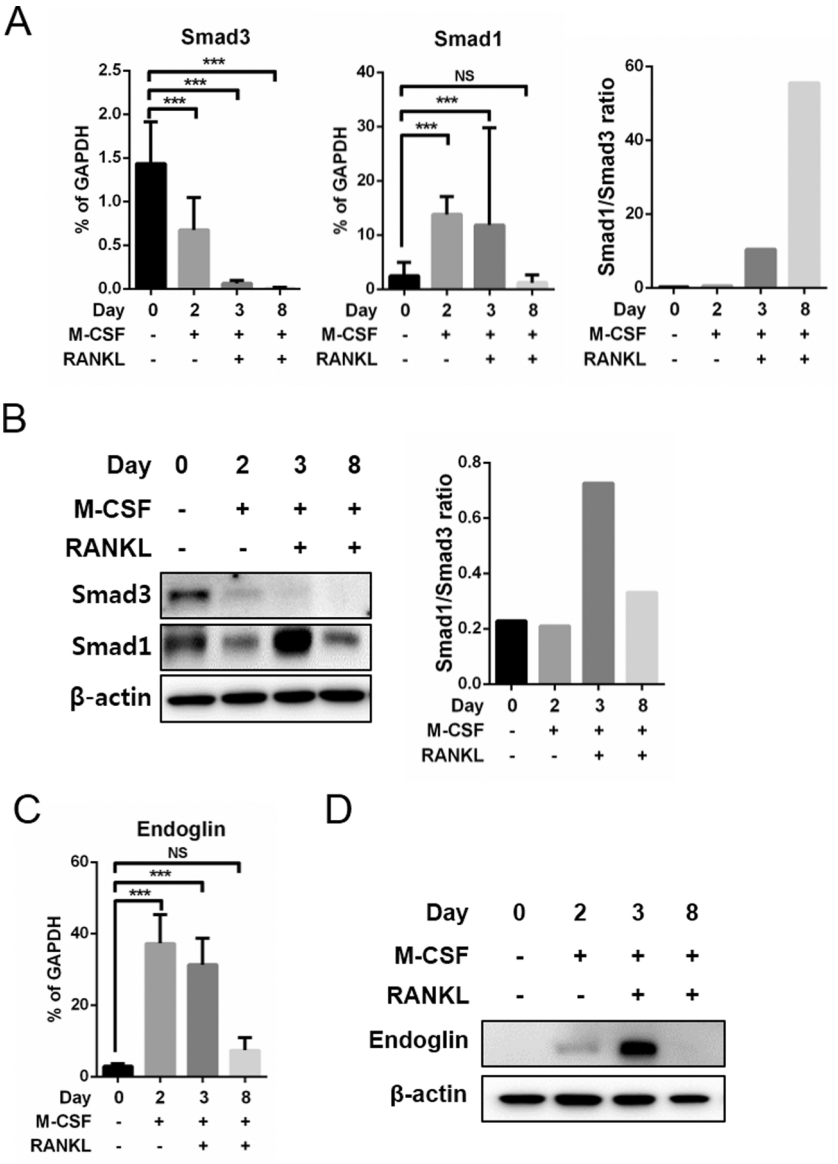

Fig. 1. Expression of SMAD1, SMAD3, and endoglin during human osteoclastogenesis. Monocytes from normal human peripheral blood (PB) were cultured with 20 ng/ml M-CSF for 2 days, after which, 40 ng/ml RANKL was added and the cells were cultured for an additional 6 days to induce osteoclast differentiation. (A) and (C) Expression of target mRNA was analyzed by qRT-PCR and normalized to that of *GAPDH*. Data are shown as means ± SD ($n = 4$); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (B) and (D) Levels of SMAD3 and SMAD1 protein were detected by western blotting. Representative blots are shown ($n = 4$).

endoglin observed was mediated by M-CSF. M-CSF R/Fc completely inhibited the induction of endoglin expression, which was increased by M-CSF (Fig. 2 C). We also tested whether RANKL affects endoglin expression during osteoclastogenesis. Isolated monocytes were cultured with M-CSF for 2 days, before being cultured in the presence or absence of RANKL for 1 day. RANKL treatment was found to induce expression of endoglin protein (Fig. 2 D).

3.2. TGF-β1 suppresses RANKL-induced osteoclastogenesis

To examine the effect of TGF-β1 on human osteoclast differentiation, we treated different concentrations of TGF-β1 (0, 0.1, 1, 10 ng/ml) during osteoclastogenesis. TGF-β1 dose-dependently reduced RANKL-induced osteoclast formation (Fig. S1). Thus, 10 ng/ml TGF-β1, which showed the most effective osteoclast inhibitory effect, was selected for further experiments. Next, we varied the timing and length of exposure to this cytokine to analyze the effective inhibitory effect timing of TGF-β1 treatment. CD14-positive cells were treated with TGF-β1 for different time intervals, as indicated in Fig. 3A. When they were exposed to TGF-β1 for the entire culture period, the number of osteoclasts was significantly reduced (Fig. 3A, B) and bone resorption decreased (Fig. 3C). Interestingly, osteoclast differentiation was also inhibited when TGF-β1 was only added during the early stage (days 1–2). However, when TGF-β1 addition was delayed, its inhibitory effect was decreased. These results indicate that TGF-β1-induced inhibition affects the early stage of osteoclast differentiation. The expression of osteoclast-related genes such as *ACP5* and *CTSK* was suppressed by TGF-β1 (Fig. 3D), and that of NFAT1, a master transcription factor in osteoclastogenesis, was also diminished by this cytokine (Fig. 3E). We subsequently investigated the influence of TGF-β1 on the RANKL signaling pathway. Pre-treatment with TGF-β1 inhibited RANKL-induced phosphorylation of p38 MAPK, but had no effect on ERK MAPK (Fig. 3F). We also found that migration of NF-κB into the nucleus was inhibited by TGF-β1, indicating that the latter inhibits RANKL-induced NF-κB activation in human osteoclasts (Fig. 3G).

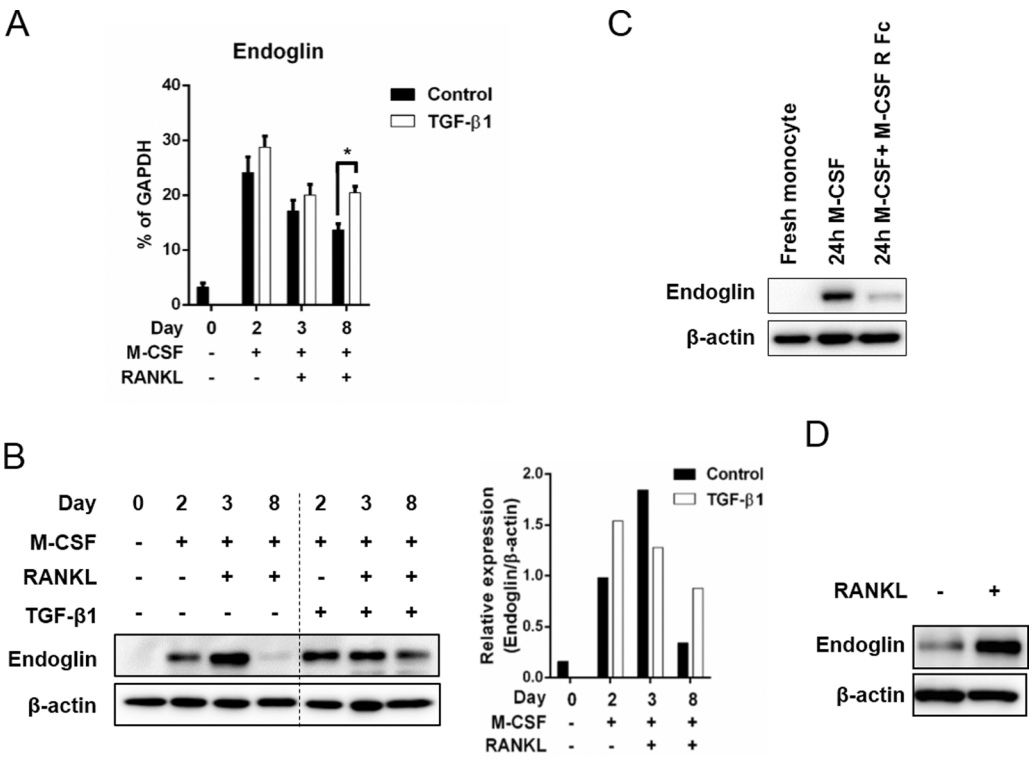

Fig. 2. Endoglin expression is induced by TGF-β1. In the presence or absence of 10 ng/ml TGF-β1, monocytes were cultured with 20 ng/ml M-CSF for 2 days, after which, 40 ng/ml RANKL was added and the cells cultured for an additional 6 days. (A) Expression of target mRNA was measured using qRT-PCR and normalized to that of *GAPDH*. Data are shown as means ± SD (n = 4); *p < 0.05 versus the control. (B) The level of endoglin protein was detected by western blotting. For the quantitative analysis of protein expression, the optical densities of blot bands were determined using image *J*. Representative data are shown (n = 3). (C) Monocytes were incubated with 20 ng/ml M-CSF for 24 h with or without M-CSF R/Fc, which blocks M-CSF signaling. The level of endoglin protein was detected by western blotting. Representative data are shown (n = 4). (D) Human osteoclast precursors were cultured with 20 ng/ml M-CSF for 24 h with or without 40 ng/ml RANKL. Whole-cell lysates were subjected to SDS-PAGE and immunoblotted. Representative data are shown (n = 3).

3.3. TGF-β1-induced SMAD1 signaling inhibits human osteoclast differentiation

A previous report has shown that phosphorylation of SMAD1/5 is significantly inhibited by 1 μM SB431542 in human macrophages [16]. In contrast, SMAD2/3 phosphorylation is inhibited by SB431542 at concentrations greater than 5 μM. Here, we verified that SB431542 inhibits TGF-β1-induced phosphorylation of SMAD1/5 at a low concentration, while leaving that of SMAD2/3 unaffected. Osteoclast precursors were starved for 6 h, treated with different concentrations of SB431542 for 30 min, and stimulated with TGF-β1 for 1 h. Phosphorylation of SMAD1 and SMAD3 was then analyzed by western blotting. SMAD1 phosphorylation was found to be reduced when SB431542 was administered at concentrations of 1 and 50 μM. In contrast, SMAD3 phosphorylation was not diminished in the presence of 1 μM SB431542, but was entirely absent at a concentration of 50 μM (Fig. 4A).

We examined osteoclast differentiation following treatment with the SMAD3 inhibitor SIS3 and ALK5 inhibitor SB431542 in order to identify the signaling pathway associated with the TGF-β1-induced inhibition of this process. We observed that the inhibition of osteoclast differentiation by TGF-β1 was reversed when 1 μM, but not 50 μM, SB431542 was administered (Fig. 4B). However, 1 μM SB431542 did not rescue the inhibitory effect of TGF-β1 on bone resorption (Fig. 4C). Following inhibition of SMAD3 activation, of which was induced by TGF-β1, using SIS3 or 50 μM SB431542, osteoclast differentiation was still inhibited by TGF-β1 treatment (Fig. 4B, C). Interestingly, SIS3 alone inhibited human osteoclast differentiation, indicating that SMAD3 activation is essential for this process (Fig. 4D).

3.4. TGF-β1 suppresses RANK and MMP9 expression in human osteoclastogenesis

We next tested whether TGF-β1 affects RANK expression, which is essential for RANKL-induced osteoclastogenesis. RANK levels were found to be reduced in the presence of TGF-β1 (Fig. 5A). TGF-β1 treatment rapidly decreased expression of *TNFRSF11A* mRNA (encoding RANK), and this reduction was partially reversed by SB431542, but not SIS3 (Fig. 5B). Similarly, inhibition of RANK protein expression by TGF-β was rescued by 1 μM SB431542, but not by SIS3 (Fig. 5C).

MMP9 expression was also inhibited by TGF-β1 (Fig. 5D, E). However, this effect was completely reversed by 50 μM SB431542 and 2.5 μM SIS3, indicating that TGF-β1-induced MMP9 inhibition is mediated by SMAD3 signaling (Fig. 5F, G). In addition, we observed that MMP9 expression was markedly suppressed by *SMAD1* siRNA, and that the inhibition of this MMP by TGF-β1 was not reversed by reducing SMAD1 expression (Fig. 5H). These results indicate that SMAD1 is essential in the expression of MMP9, rather than being involved in its inhibition by TGF-β1.

4. Discussion

In this study, we have shown that TGF-β has both inhibitory and stimulatory effects during human osteoclastogenesis, and that these opposing functions are mediated by SMAD1 and SMAD3 signaling, respectively. We also examined the mechanism underlying TGF-β-mediated inhibition of human osteoclastogenesis. TGF-β1 downregulated RANK expression and inhibited RANKL-induced nuclear translocation of NF-κB. Our findings identify a regulatory function for TGF-β1 signaling in human osteoclastogenesis.

A recent study reported that TGF-β1 activates the SMAD1/5 pathway in human macrophages via ALK5 [16]. This investigation employed SB431542, an inhibitor of ALK4/5/7, providing a unique opportunity to dissect SMAD1/5- and SMAD2/3-dependent TGF-β-induced signaling. Low-dose SB431542 completely inhibited SMAD1/5 phosphorylation, while leaving sufficient levels of SMAD2/3 phosphorylation. High doses, however, inhibited activation of both SMAD1/5 and SMAD2/3 [16]. In human osteoclast precursors, we observed that SIS3 and low- and high-dose SB431542 inhibited SMAD3, SMAD1, and both SMAD1 and SMAD3, respectively. SIS3 and 50 μM SB431542 inhibited osteoclast differentiation; thus, SMAD3 is expected to be involved in promoting this process, consistent with the results of previous studies [10,11]. On the other hand, the inhibition of osteoclast differentiation by TGF-β1 was reversed in the presence of 1 μM SB431542, which inhibits SMAD1 activation. Therefore, TGF-β-mediated

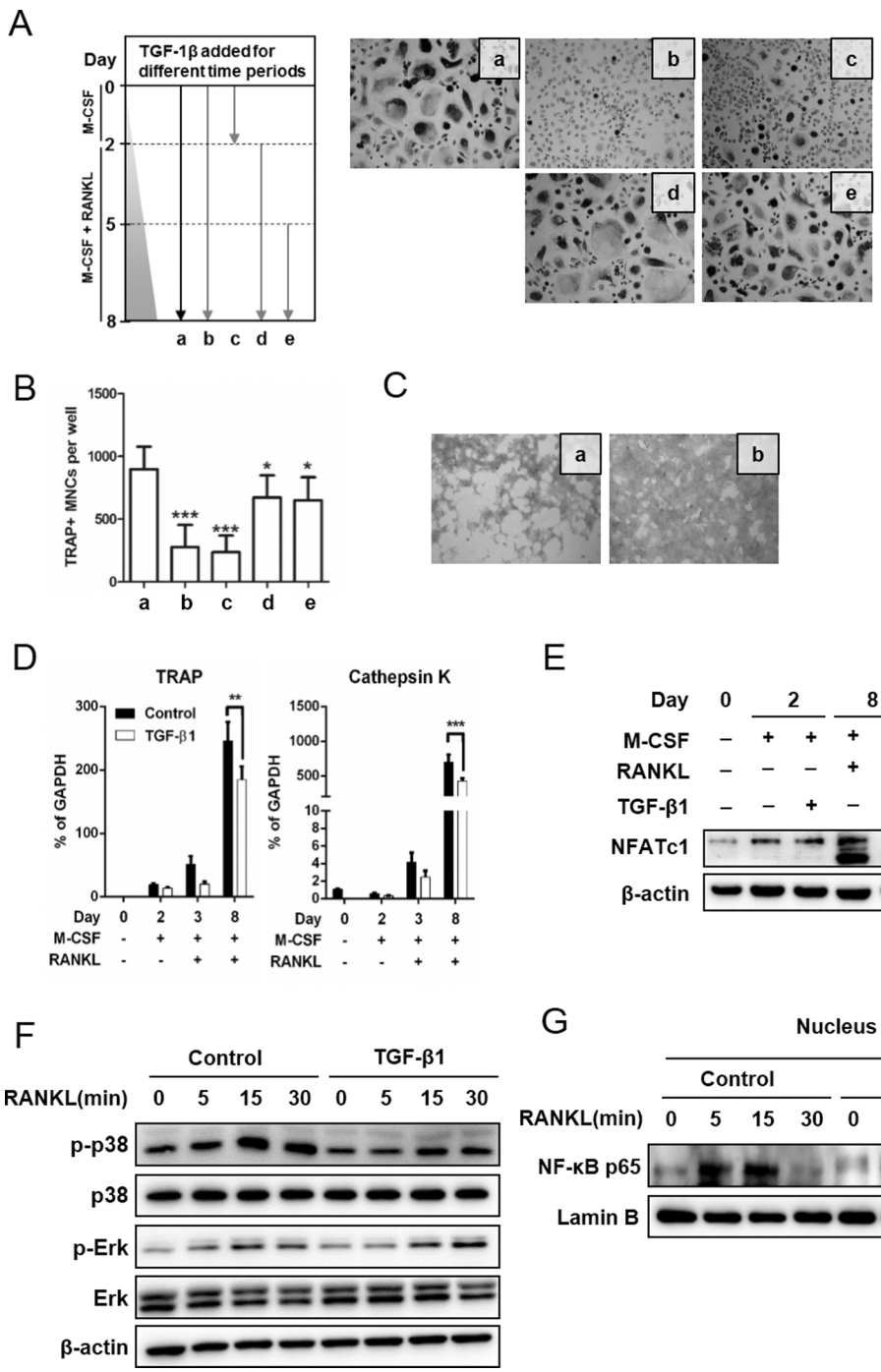

Fig. 3. TGF-β1 suppresses RANKL-induced osteoclastogenesis and signaling. (A) and (B) In the absence (a) or presence (b–e) of 10 ng/ml TGF-β1 for the indicated time, monocytes were cultured with 20 ng/ml M-CSF for 2 days, after which, 40 ng/ml RANKL was added and the cells cultured for an additional 6 days. The cells were stained for ACP5 (also known as TRAP) on day 8. The number of multinucleated (> 3 nuclei/cell) ACP5-positive cells, considered to be osteoclasts, was recorded (original magnification: 100×). Data are shown as means ± SD ($n = 5$); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (C) In the presence or absence of 10 ng/ml TGF-β1, monocytes were cultured with 20 ng/ml M-CSF for 2 days, after which, 40 ng/ml RANKL was added and the cells cultured for an additional 6 days. The osteoclast bone resorption assay was performed by using a bone resorption assay kit, with the resorbed areas on the plate being visualized by light microscopy (original magnification: 100×). (D) In the presence or absence of 10 ng/ml TGF-β1, monocytes were cultured with 20 ng/ml M-CSF for 2 days, after which, 40 ng/ml RANKL was added and the cells cultured for an additional 6 days. Expression of target mRNA was measured using qRT-PCR and normalized to that of *GAPDH*. Data are shown as means ± SD ($n = 4$); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (E) Cells were cultured as in (D). Whole-cell lysates were subjected to SDS-PAGE and immunoblotted. Representative data are shown ($n = 5$). (F) and (G) Human monocytes were cultured with 20 ng/ml M-CSF for 2 days with or without 10 ng/ml TGF-β1. The cells were serum-starved for 6 h and thereafter stimulated with 40 ng/ml RANKL for the indicated time. Whole-cell lysates and nuclear extracts were subjected to SDS-PAGE and immunoblotted. Representative data are shown ($n = 5$).

suppression of osteoclast differentiation acts through SMAD1-dependent signaling. Interestingly, although the lower dose of SB431542 used mitigated the inhibitory effect of TGF-β on osteoclast differentiation, it did not reverse the inhibition of bone resorption, which may therefore be induced by signaling pathways other than those involving SMAD1.

The ratio of the expression of SMAD1 and SMAD3, TGF-β1-induced signaling molecules, was determined during osteoclast differentiation. The SMAD1/SMAD3 ratio was found to increase at both the mRNA and protein level upon osteoclast formation (Fig. 1A, B). In addition, the effect of SMADs is influenced not only by protein expression level but also by the degree of activation (phosphorylation). In our experiment, we observed the phosphorylation of SMAD3 induced by TGF-β was lower in Day 2 than that of Day 0 in some cases, but not in all experiments (Fig. S2).

Endoglin is a transmembrane accessory receptor of TGF-β, and recent studies have reported that in endothelial cells and chondrocytes, it differentially regulates TGF-β-induced SMAD2/3 and SMAD1/5 signaling depending on cellular differentiation state [17–19]. In our study, endoglin was observed to be expressed on osteoclast precursors and immature osteoclasts and its expression was induced by M-CSF (Fig. 2C) and RANKL (Fig. 2D), crucial cytokines in osteoclast differentiation. Furthermore, endoglin levels were increased by TGF-β (Fig. 2A, B). Taken together, these results suggest that endoglin may be involved in the inhibition of TGF-β-induced osteoclast differentiation by regulating the balance between SMAD1 and SMAD3 signaling. However, further studies are needed through knockout experiments to confirm the role of endoglin in osteoclast differentiation.

RANK is a critical receptor in osteoclast differentiation [20]. RANK

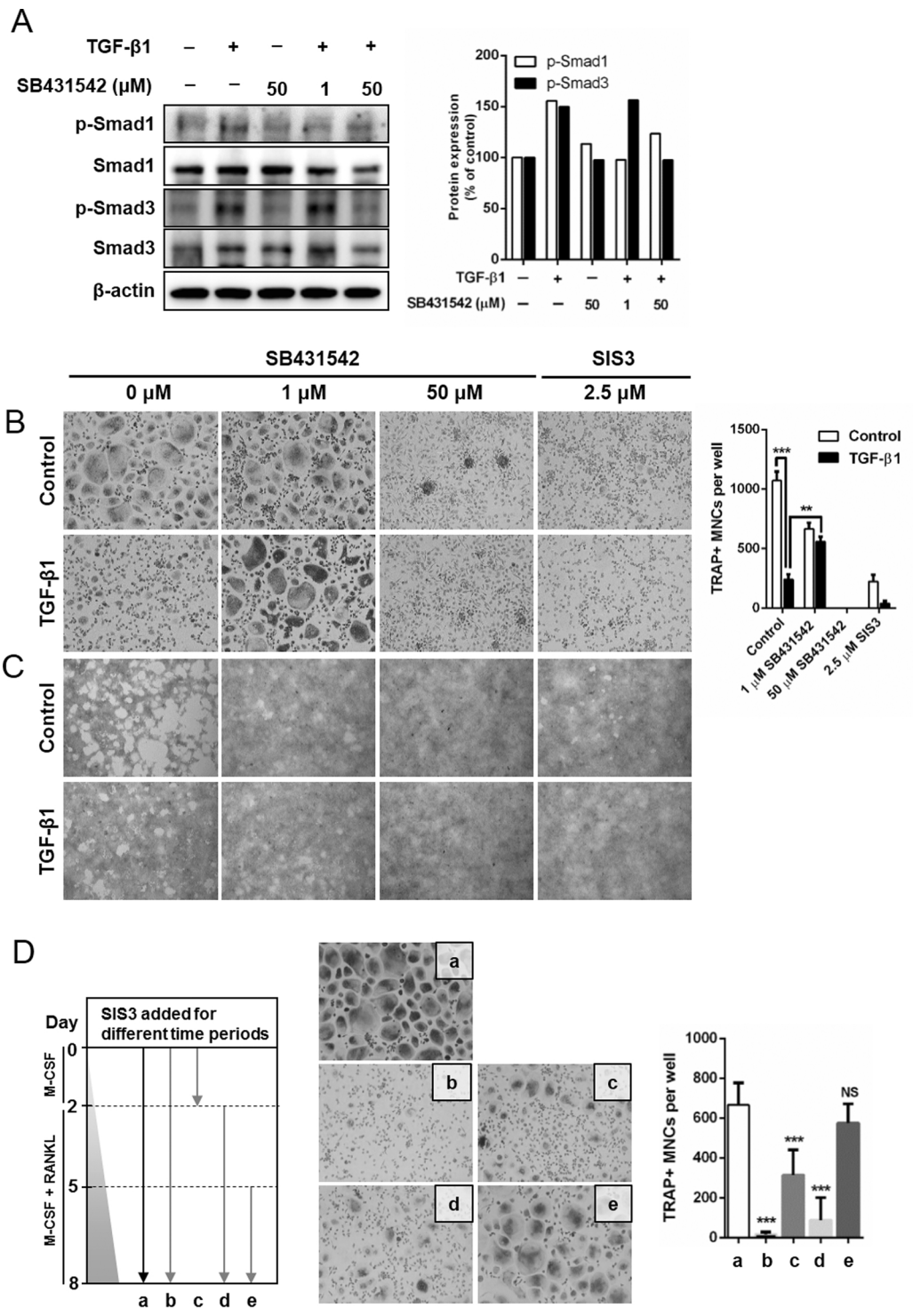

Fig. 4. TGF-β1-induced SMAD1 signaling inhibits osteoclast differentiation. (A) Human osteoclast precursors were stimulated with 10 ng/ml TGF-β1 for 30 min, with SB431542 (1 or 50 μM) having been added 30 min beforehand. Whole-cell lysates were subjected to SDS-PAGE and immunoblotted. Representative data are shown ($n = 5$). (B) In the presence or absence of 10 ng/ml TGF-β1, monocytes were cultured with 20 ng/ml M-CSF for 2 days, after which, 40 ng/ml RANKL was added and the cells cultured for an additional 6 days. SB431542 (1 or 50 μM) or SIS3 (2.5 μM) was added together with M-CSF during osteoclastogenesis. Cells were stained for ACP5 (also known as TRAP) expression. The number of multinucleated (> 3 nuclei/cell) ACP5-positive cells, considered to be osteoclasts, was recorded. Data are shown as means ± SD ($n = 5$); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (C) Cells were cultured as in (B). The osteoclast bone resorption assay was performed using a bone resorption assay kit, with the resorbed areas being visualized by light microscopy (original magnification: 100×). (D) In the absence (a) or presence (b–e) of 2.5 μM SIS3 for the indicated time, monocytes were cultured with 20 ng/ml M-CSF for 2 days, after which, 40 ng/ml RANKL was added and the cells cultured for an additional 6 days. The number of multinucleated (> 3 nuclei/cell) ACP5-positive cells, considered to be osteoclasts, was recorded (original magnification: 100×). Data are shown as means ± SD ($n = 5$); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

expression is regulated by TGF-β1, being increased or decreased depending on the cell type and species concerned [7]. In RAW 264.7 murine monocytes, TGF-β1 stimulates osteoclast differentiation by upregulating RANK expression. We observed that the SMAD3 inhibitor SIS3 reduces RANK levels, indicating that SMAD3 may increase expression of this receptor. On the other hand, TGF-β1 inhibits osteoclastogenesis in humans via downregulation of RANK [12]. In our study, SMAD1 signaling was found to be involved in the inhibition of RANK expression, since 1 μM SB431542 reversed TGF-mediated RANK downregulation. Our results thus suggest that SMAD3 and SMAD1 signaling respectively induces and suppresses RANK expression in human osteoclast precursors.

The activation of NF-κB is essential in RANKL-induced osteoclast differentiation. NF-κB proteins reside in the cytoplasm of non-stimulated cells, but rapidly enter the nucleus upon stimulation of cells with RANKL during osteoclastogenesis [21]. However, pre-treatment with TGF-β1 suppressed RANKL-induced NF-κB nuclear translocation in the present work. TGF-β inhibits NF-κB activity through induction of IκB-α expression in human salivary gland cells and in human fibroblast-like synoviocytes, it suppresses the degradation of cytosolic IκB-α and the translocation of activated NF-κB to the nucleus [22]. Moreover, TGF-β pretreatment restricts IκB-α phosphorylation in the intestinal

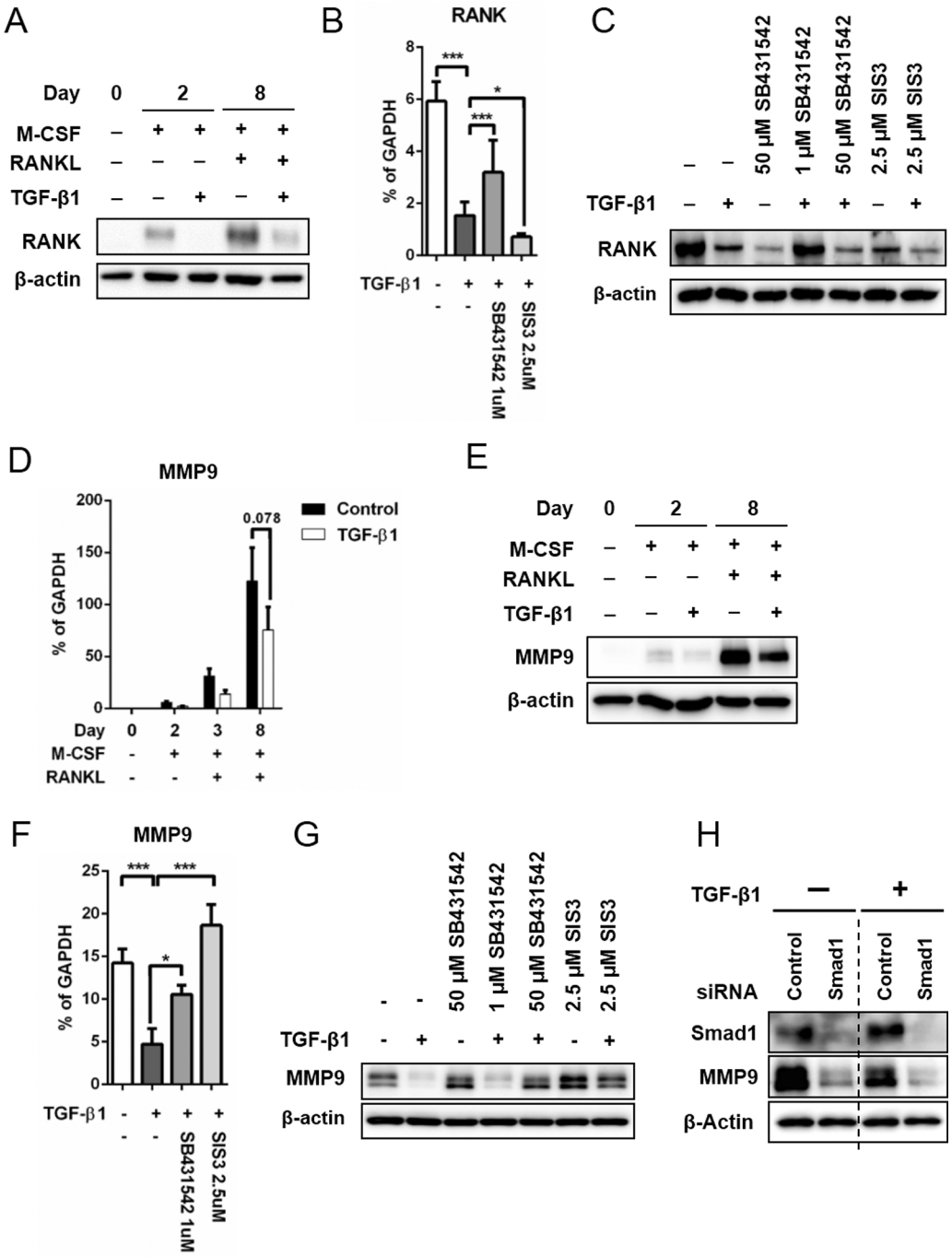

Fig. 5. TGF-β1 suppresses RANK and MMP9 expression in human osteoclastogenesis. (A) In the presence or absence of 10 ng/ml TGF-β1, monocytes were cultured with 20 ng/ml M-CSF for 2 days, after which, 40 ng/ml RANKL was added and the cells cultured for an additional 6 days. Whole-cell lysates were subjected to SDS-PAGE and immunoblotted. Representative data are shown ($n = 5$). (B) and (F) Monocytes were cultured with 20 ng/ml M-CSF with or without 10 ng/ml TGF-β1 for 2 days, with 1 μM SB431542 or 2.5 μM SIS3 having been added 30 min beforehand. Expression of target mRNA was measured using qRT-PCR and normalized to that of *GAPDH*. Data are shown as means ± SD ($n = 4$); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (C) and (G) Monocytes were cultured with 20 ng/ml M-CSF with or without 10 ng/ml TGF-β1 for 2 days, with SB431542 (1 or 50 μM) or SIS3 (2.5 μM) having been added 30 min beforehand. Whole-cell lysates were subjected to SDS-PAGE and immunoblotted. Representative data are shown ($n = 4$). (D) In the presence or absence of 10 ng/ml TGF-β1, monocytes were cultured with 20 ng/ml M-CSF for 2 days, after which, 40 ng/ml RANKL was added and the cells cultured for an additional 6 days. Expression of target mRNA was measured using qRT-PCR and normalized to that of *GAPDH*. Data are shown as means ± SD ($n = 4$); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (E) Immunoblot of MMP9 expression in cells treated with TGF-β1. Representative data are shown ($n = 4$). (H) Monocytes were transfected with *SMAD1*-targeting siRNA or control siRNA. Knockdown was verified at the protein level by western blotting for SMAD1. Representative data are shown ($n = 3$).

epithelium [23]. We observed that IκB-α expression was induced by TGF-β in some cases, but not consistently (Fig. S3). However, it remains possible that the inhibition of NF-κB nuclear translocation by TGF-β is regulated via IκB-α.

MMP9 is highly expressed in osteoclasts and plays an important role in the degradation of the extracellular matrix [24]. In human monocytes, TGF-β downregulates MMP9 [25], and this effect is attenuated in *Smad3*-null mice [26]. Moreover, MMP12 is also inhibited by TGF-β-induced SMAD3 expression in human macrophages [27]. We also confirmed here that TGF-β decreases MMP9 expression in RANKL-induced osteoclast formation, as noted in previous studies [25]. Further, we found that the inhibitory effect of TGF-β on MMP9 was reversed by SIS3, a SMAD3 inhibitor, confirming SMAD3-dependent regulation. Interestingly, when SMAD1 was downregulated using siRNA, MMP9 levels were more markedly diminished, indicating that SMAD1 is an important molecule for the induction of MMP9 expression in osteoclasts. Our results suggest a model that TGF-β inhibits and stimulates human osteoclast differentiation and function by SMAD1 and SMAD3 signaling, respectively. (Fig. S4).

In conclusion, this study showed that TGF-β has both inhibitory and stimulatory effects on human osteoclast differentiation, and that these opposing functions are mediated by SMAD1 and SMAD3 signaling, respectively.

Conflict of interest statement

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2018.12.003.

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