C18 ORF1, a Novel Negative Regulator of Transforming Growth Factor-\(\beta\) Signaling

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Background: The structure of C18ORF1 is similar to that of TMEPAI.

Results: C18ORF1 inhibits TGF-\(\beta\) signaling, but not BMP signaling, by its competition with SARA for Smad2/3 binding.

Conclusion: C18ORF1 is a surveillant during the steady state of TGF-\(\beta\) signaling, although it is helped by TMEPAI to inhibit TGF-\(\beta\) signaling in a coordinated manner.

Significance: C18ORF1 acts as a gatekeeper that abrogates excessive TGF-\(\beta\) signaling.

Transforming growth factor (TGF)-\(\beta\) signaling is deliberately regulated at multiple steps in its pathway from the extracellular microenvironment to the nucleus. However, how TGF-\(\beta\) signaling is activated or attenuated is not fully understood. We recently identified transmembrane prostate androgen-induced RNA (TMEPAI), which is involved in a negative feedback loop of TGF-\(\beta\) signaling. When we searched for a family molecule for TMEPAI, we found C18ORF1, which, like TMEPAI, possesses two PY motifs and one Smad-interacting motif (SIM) domain. As expected, C18ORF1 could block TGF-\(\beta\) signaling but not bone morphogenetic protein signaling. C18ORF1 bound to Smad2/3 via its SIM and competed with the Smad anchor for receptor activation for Smad2/3 binding to attenuate recruitment of Smad2/3 to the TGF-\(\beta\) type I receptor (also termed activin receptor-like kinase 5 (ALK5)), in a similar fashion to TMEPAI. Knockdown of C18ORF1 prolonged duration of TGF-\(\beta\)-induced Smad2 phosphorylation and concomitantly potentiated the expression of JunB, p21, and TMEPAI mRNAs induced by TGF-\(\beta\). Consistently, TGF-\(\beta\)-induced cell migration was enhanced by the knockdown of C18ORF1. These results indicate that the inhibitory function of C18ORF1 on TGF-\(\beta\) signaling is similar to that of TMEPAI. However, in contrast to TMEPAI, C18ORF1 was not induced upon TGF-\(\beta\) signaling. Thus, we defined C18ORF1 as a surveillant of steady state TGF-\(\beta\) signaling, whereas TMEPAI might help C18ORF1 to inhibit TGF-\(\beta\) signaling in a coordinated manner when cells are stimulated with high levels of TGF-\(\beta\).

Transforming growth factor-\(\beta\) (TGF-\(\beta\)) is a multifunctional cytokine that regulates the growth, differentiation, motility, apoptosis, and matrix protein production of a number of cell types (1, 2). The TGF-\(\beta\) family starts to transduce its signals via specific serine/threonine kinase receptors on the cell membrane. Then the canonical and noncanonical TGF-\(\beta\) signaling pathways into the cell are activated. In brief, after TGF-\(\beta\) binds to its type II receptor, the complex recruits TGF-\(\beta\) type I receptor (activin receptor-like kinase 5 (ALK5)) to make a ternary complex. Subsequently, TGF-\(\beta\) type II receptor kinase phosphorylates ALK5 in its juxtamembrane domain, termed the GS domain, to activate it. In the canonical TGF-\(\beta\) pathway, the adaptor proteins Smad anchor for receptor activation (SARA) and hepatocyte growth factor-regulated tyrosine kinase substrate/(Hgr/Hrs) recruit the activin/TGF-\(\beta\) receptor-regulated Smads (AR-Smads; i.e. Smad2 and Smad3) to the active ALK5, which phosphorylates AR-Smads at two serine residues located at their extreme C terminus. The two phosphorylated AR-Smads then make a heteromeric complex with one Smad4 to translocate to the nucleus, where this AR-Smads/Smad4 complexes function as a transcriptional corepressor.

The abbreviations used are: SARA, Smad anchor for receptor activation; TMEPAI, transmembrane prostate androgen-induced RNA; SIM, Smad-interacting motif; BMP, bone morphogenetic protein; PLA, proximity ligation assay; MEF, mouse embryonic fibroblast; SBE, Smad-binding element; EMT, epithelial-mesenchymal transition; AR-Smad, activin/TGF-\(\beta\) receptor-regulated Smad; SARA(SBD), SARA(Smad binding domain).
nary complex transcriptionally regulates its target genes (3). However, the TGF-β pathways that do not transduce signals via Smads act as noncanonical TGF-β pathways, which include the p38, JNK, P38K, Par6, and Rho pathways. These noncanonical TGF-β pathways are known to occasionally compensate the Smad pathway (3–8). Because the TGF-β family plays crucial roles in embryogenesis and maintenance of tissue homeostasis during adult life, a number of lines of evidence indicate that dysregulation of TGF-β signaling contributes to various disorders, including cancer, fibrosis, and vascular disorders (9, 10). To prevent excessive TGF-β signaling in cells, TGF-β signaling is tightly regulated at multiple steps from the extracellular microenvironment to the nucleus, including by entrapment of Smads to terminate TGF-β-Smad interactions. Thus, active ALK5 cannot be provided with AR-Smad interaction.

Previously, we reported that TMEPAI, a direct target gene of TGF-β/activin signaling, inhibits TGF-β/activin signaling through a negative feedback loop. This inhibitory action of TMEPAI is due to its competition with SARA for binding to AR-Smads. Thus, active ALK5 cannot be provided with AR-Smads to terminate TGF-β/activin signaling (13). Because TMEPAI possesses unique motifs in its cytoplasmic region (two PY motifs that can interact with WW domain-containing proteins and one Smad-interacting motif (SIM) that can recognize AR-Smads), we searched for a TMEPAI family molecule(s) that also has a PY motif and SIM. Our screening yielded C18ORF1. C18ORF1 has a low density lipoprotein receptor class A domain-containing protein 4 in its extracellular domain and is a putative schizophrenia-related gene (14–16). However, its mechanism of action and physiological function are still unclear. Here, we show that C18ORF1 can specifically inhibit TGF-β signaling in a similar fashion to TMEPAI as a gatekeeper that abrogates excessive TGF-β signaling.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids**—Human C18ORF1 cDNA was cloned by RT–PCR. All of the C18ORF1 mutants were made by using a QuickChange site-directed mutagenesis kit (Stratagene) or PrimeStar HS DNA polymerase (Takara Bio). C18ORF1 and its mutants were inserted into pcDNA3.1-V5-His-A (Invitrogen), pcDNA3-HA, or pcDNA3-FLAG (17). All C18ORF1 constructs possessed the FLAG, HA, or V5 epitope tag at their C terminus. Adenoviruses expressing C18ORF1/V5 or C18ORF1(4A)/V5 were generated using the pAdTrack-CMV vector. After recombination of either pAdTrack-CMV-C18ORF1/V5 or pAdTrack-CMV-C18ORF1(4A)/V5 with pAdEasy-1 (18), the resulting plasmids were transfected into 293Ter cells, and the adenoviruses were amplified. The other constructs were previously described (13, 19–24).

**Antibodies**—Antibodies were obtained from the following sources: mouse monoclonal anti-FLAG M2, anti-FLAG M5, and anti-β-actin antibodies from Sigma; mouse polyclonal C18ORF1 antibody from Abnova; mouse monoclonal anti-p21, anti-Myc9E10, and anti-GFP antibodies from Santa Cruz Biotechnology; rat monoclonal anti-HA 3F10 antibodies from Roche Applied Science; mouse monoclonal V5 antibodies from Invitrogen; mouse monoclonal anti-E-cadherin and anti-Smad2/3 antibodies from BD Transduction Laboratories; and rabbit monoclonal anti-Smad3 antibody from Cell Signaling Technology. Rabbit polyclonal phosphorylated Smad2 and Smad1/3 antibodies were in-house (25).

**Cell Culture**—NMuMG, HaCaT, 911, 293, HeLa, A549, and COS7 cells were cultured in Dulbecco’s modified Eagle’s medium (Nacalai Tesque) containing 10% fetal calf serum (FCS; Invitrogen). HepG2 cells were maintained in minimum essential medium (Wako) containing 10% FCS, nonessential amino acids (Nacalai Tesque), and sodium pyruvate. Mouse embryonic fibroblasts (MEFs) from C18ORF1 and TMEPAI knock-out mice were prepared and cultured in DMEM containing 10% FCS.

**Transcriptional Reporter Assays**—One day before transfection, HepG2 cells were seeded at 1.0 × 10⁵ cells/well in 12-well plates. The cells were transfected using polyethyleneimine (Polysciences). Where indicated, 5 ng/ml TGF-β or 25 ng/ml BMP-6 was added to the wells 24 h after transfection. Subsequently, the cells were cultured in the absence of FCS for 18 h. In all experiments, β-galactosidase (pCH110; GE Healthcare) activity was measured to normalize for transfection efficiency. Each transfection was carried out in triplicate and repeated at least twice.

**Immunoprecipitation and Western Blot Analysis**—To detect interactions among proteins, plasmids were transfected into COS7 cells (5 × 10⁵ cells/6-cm dish) using polyethyleneimine. Forty hours after transfection, cells were lysed in 500 μl of TNE buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM PMSF, 5 μg/ml leupeptin, 100 units/ml aprotinin, 2 mM sodium vanadate, 40 mM NaF, and 20 mM β-glycerophosphate). Cell lysates were precleared with protein G-Sepharose beads (GE Healthcare) for 30 min at 4 °C and then incubated with either anti-FLAG M5 or anti-FLAG M2 antibody for 2 h at 4 °C. Protein complexes were immunoprecipitated by incubation with protein G-Sepharose beads for 30 min at 4 °C and then washed three times with TNE buffer. Immunoprecipitated proteins and aliquots of total cell lysates were boiled for 5 min in sample buffer, separated by SDS-PAGE, and transferred to Hybond-C Extra membranes (GE Healthcare). The membranes were probed with primary antibodies. The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and chemiluminescent substrate (Thermo Scientific). Protein expression in total cell lysates was evaluated by Western blot analysis.

**RNA Preparation and RT–PCR**—Total RNA was extracted using an RNeasy Plus mini kit (Qiagen). Reverse transcription was performed with a High-Capacity RNA-to-cDNA kit (Applied Biosystems). PCR was performed using GoTaq (Promega) according to the manufacturer’s instructions. To distinguish mRNA expressions among human C18ORF1α, C18ORF1β, and C18ORF1γ, three primer sets were synthesized as follows: 5’-ATGCCGAAGAAGGTGTCTTCTACA’-3’ and 5’-CGATGATGATGATTGGCG-3’ for C18ORF1α; 5’-AGGAACAGCGTGTTAGA’-3’ and 5’-CGATGATGATGTTGGCG-3’ for C18ORF1β; and 5’-ATAACTGACGCTCTGAGCTG-3’ and 5’-CGATGATGATGATTGGCG-3’ for C18-

*4 S. Itoh, Y. Watanabe, F. Itoh, S. Takahashi, and M. Kato, unpublished results.*
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ORF1γ. In addition, total expression of human C18ORF1 mRNAs was detected using one set of primers (5'-CGCCCACA-TATCTCATTGAC-3' and 5'-AAGCGATCCCTCTGGATGAA-3'). The following primer sets were used to amplify TMEPAI, C18ORF1, SMAD6, JUNB, and β-ACTIN cDNAs: 5'-CAAGAGATGCGACCTCAGTAC-3' and 5'-CTCCTCTCGATTCGGA-3' for human TMEPAI; 5'-CGAGA-GCTAAAACGGTCAC-3' and 5'-AATTCCCAGGGAGCTGTA-3' for human SMAD6; 5'-ACCTCTCTCTACGACT-3' and 5'-GCTTGTTTCATCTTGTGCA-3' for human JUNB; 5'-CAAGAGATGCGACCTCAGTAC-3' and 5'-CTCCTCTCGATTCGGA-3' for human β-ACTIN; 5'-CGAGCA-GATCCTTATCATGG-3' and 5'-GAATGGATCCCTCNTGATGA-3' for mouse C18orf1; 5'-GTGATGATGTTGTGATGTTGG-3' and 5'-ATCACAGCTAGATGATGTTGG-3' for mouse Tmepai; and 5'-GCTCATAGCTTCTCAGGGA-3' and 5'-TGAACCTTAAGGCAACCGTT-3' for mouse β-ACTin.

Quantitative Real Time-PCR Analysis—Preparation of total RNA, reverse transcription, and oligonucleotide DNA primers were described above. Quantitative PCR was performed with FastStart SYBR Green Master mix (Roche Applied Science). All reactions were carried out on a StepOne Plus (ABI). Each sample was analyzed in triplicate for each PCR measurement. Melting curves were checked to ensure specificity. Relative quantification of mRNA expression was calculated using the standard curve method with β-actin level.

Proximity Ligation Assay (PLA)—Cells on cover glasses coated with 0.1% gelatin were cultured with DMEM. Then the cover glasses were washed once with phosphate-buffered saline (PBS), fixed for 10 min with 4% paraformaldehyde (Wako), washed three times with PBS, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and again washed three times with PBS.

The following procedures were performed according to the manufacturer's instructions (Olink Bioscience). To visualize the fluorescence, an immunofluorescence microscope (Nikon) was used.

Immunofluorescence Staining—Immunofluorescence staining was performed as described previously (13). Briefly, cells grown on the cover glasses were transfected with the indicated plasmids. If necessary, cells were stimulated with TGF-β for 2 h. After treatment, the dishes were washed once with PBS, fixed for 10 min with 4% paraformaldehyde, washed three times with PBS, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and again washed three times with PBS. The cover glasses were blocked with 5% normal swine serum (Dako) in PBS at 37 °C for 1 h and incubated with 5% normal swine serum (in PBS) containing mouse monoclonal anti-V5 and rat monoclonal anti-HA3F10 antibodies at 4 °C overnight. The cover glasses were then washed three times with PBS, incubated with 5% normal swine serum (in PBS) containing both FITC-conjugated goat anti-mouse IgG antibody (diluted 1:250) (Invitrogen) and Texas Red-conjugated goat anti-rabbit IgG antibody (diluted 1:250) (Invitrogen) at room temperature for 1 h, and washed three times with PBS. Nuclei were stained with DAPI. To visualize the fluorescence, an immunofluorescence microscope was used.
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Structure of C18orf1α1 (the longest form of C18orf1) with that of TMEPAI revealed that similarity between C18orf1α1 and TMEPAI was 75 and 67% in the transmembrane and intracellular domains, respectively (Fig. 1B). However, the extracellular domains of C18orf1α1 and TMEPAI have low similarity. The other five C18orf1 isoforms also showed low homology with TMEPAI in their extracellular domains, which diverge among the C18orf1 subfamily. TMEPAI has been reported to inhibit TGF-β signaling via its SIM domain (13). All of the C18orf1 isoforms include the SIM domain in their structures (Fig. 1A). Thus, we speculated that C18orf1 might act as a negative regulator of TGF-β signaling, like TMEPAI (see below). Among the six C18orf1 isoforms, the C18orf1γ subfamily was not detected in cells, whereas the transcript of the C18orf1α subfamily in the subsequent experiments.

FIGURE 1. Comparison between C18orf1 and TMEPAI. A, alternative splicing forms of C18orf1. C18orf1 has six isoforms. TM, transmembrane domain; PY, PY motif; SIM, Smad-interacting motif. β, alignment of amino acid sequences between C18orf1α1 and TMEPAI. C, expression of C18orf1 isoforms detected by RT-PCR. Total RNAs from HepG2, 293, and HeLa cells were prepared and followed by cDNA synthesis and PCR. Upper panel, whole C18orf1 expression is shown. The upper and lower bands indicate C18orf1 with and without 18 amino acids, respectively. The 2nd, 3rd, 4th, and bottom panels show expressions of C18orf1α1, C18orf1β, and C18orf1γ subfamily, and β-actin mRNAs, respectively. D, effect of TGF-β or BMP on C18orf1 mRNA expression. HepG2 cells were stimulated with either 5 ng/ml TGF-β or 25 ng/ml BMP-6 for indicated times. The RT-PCR was then performed using specific primer sets. The upper and lower bands corresponding to C18orf1 mRNA indicate C18orf1 and C18orf1 without 18 amino acids in its cytoplasmic region, respectively. TMEPAI and Smad6 mRNA expressions were used as positive controls for TGF-β and BMP-6 stimulation, respectively. β-Actin mRNA was used as an internal control. E, expression of C18orf1 and TMEPAI by quantitative PCR. All values represent mean ± S.D. Significantly different from the absence of TGF-β; *, p < 0.05; **, p < 0.01; ***, p < 0.001. F, localization of C18orf1 with TMEPAI. Either C18orf1α1/V5 (left panels) or C18orf1α2/V5 (right panels) was transfected with TMEPAI/HA into 911 cells. Twenty four hours after transfection, cells were fixed and stained with mouse anti-V5 and rat anti-HA3F10 monoclonal antibodies. The Alexa488-conjugated goat anti-mouse and the Alexa555-conjugated goat anti-rat IgG antibodies (Molecular Probes) were used for visualization. Colocalization in the merge panel can be seen in yellow. Nuclear staining (blue) was carried out using DAPI. G, TMEPAI makes homomeric interaction as well as heteromeric interaction with C18orf1α1. The cell lysates were immunoprecipitated (IP) with anti-FLAG M2 antibody and then analyzed by Western blot (WB) with anti-V5 antibody (upper panels). The middle and lower panels indicate total expressions of TMEPAI/V5 and C18orf1α1/FLAG or TMEPAI/FLAG, respectively. H, homomeric and heteromeric complex between C18orf1α1 and TMEPAI. The experiments were carried out according to the description above. Upper panel, heteromeric complex between C18orf1α1 and TMEPAI or homomeric complex of C18orf1α1. Middle panel, expression of C18orf1α1/V5. Lower panel, expression of TMEPAI/FLAG and C18orf1α1/FLAG. J, profile of C18orf1 mRNA expression in mouse tissues. Upper panel, expression of mouse C18orf1 mRNA (middle panel) expression of mouse Tmeap1 mRNA (lower panel) expression of mouse β-actin mRNA.

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Because TMEPAI is a direct target gene of TGF-β signaling (13, 24), we investigated whether the C18ORF1 transcript was augmented in cells upon TGF-β stimulation. However, in contrast to the TMEPAI transcript, the expression of C18ORF1 mRNA was only marginally induced by TGF-β but not by BMP (Fig. 1, D and E). We have already revealed that TMEPAI localizes to early endosomes together with SARA (13). Hence, we examined whether C18ORF1 can be colocalized with TMEPAI in cells. In fluorescence immunostaining, C18ORF1α1 and C18ORF1α2 deficient in 18 amino acids between the transmembrane and SIM domains were cotransfected with TMEPAI and C18ORF1α1 on 911 cells. Both TMEPAI and C18ORF1 showed colocalization with typical punctate staining (Fig. 1F). Thus, C18ORF1 is probably present in early endosomes. Consistently, C18ORF1α1

FIGURE 2. C18ORF1 inhibits TGF-β signaling. A and B, effect of C18ORF1 on TGF-β or BMP-induced luciferase reporter activity. HepG2 cells were transfected with indicated plasmids at different doses. Twenty-four hours later, cells were stimulated with either 5 ng/ml TGF-β (A) or 25 ng/ml BMP-6 (B). All values represent mean ± S.D. Significantly different from mock in the presence of TGF-β: *, p < 0.05; **, p < 0.01. C and D, additional inhibitory effect of TMEPAI family on TGF-β-induced reporter activity. HepG2 cells carrying a certain amount of TMEPAI (C) and C18ORF1 (D) were transfected with increased amounts of C18ORF1 and TMEPAI, respectively. Twenty-four hours later, cells were stimulated with either 5 ng/ml TGF-β. All values represent mean ± S.D. Significantly different from mock in the presence of TGF-β: ***, p < 0.001.

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could also physically interact with TMEPAI, both of which made a homomeric complex (Fig. 1, G and H). When we checked the expression of Tmepai and C18orf1 in mouse tissues, C18orf1 mRNA could be detected in almost all tissues examined, whereas Tmepai mRNA could not be seen in some of tissues. Thus, the mRNA expression profiles between C18orf1 and Tmepai were not completely identical in tissues (Fig. 1f).

Inhibitory Effect of C18orf1 on TGF-β Signaling—Because the primary protein structure of C18orf1 in its intracellular domain is highly similar to that of TMEPAI, we tested the effect of C18orf1 on the Smad-driven transcriptional (SBE)₄-luc reporter (20). Both C18orf1α1 and C18orf1α2 could perturb TGF-β-induced reporter activity in a dose-dependent manner, although they did not affect BMP-induced reporter activity (Fig. 2, A and B). Because both C18orf1 and TMEPAI can make a heterodimer as well as a homodimer, we explored whether the TMEAPI family could inhibit TGF-β signaling either synergistically or additionally. Fig. 2, C and D, indicated that inhibition of TGF-β signaling by the TMEPAI family might be an additional effect rather than a synergistic effect. We also performed luciferase assays using MEFs from either C18orf1 or Tmepai knock-out mice (Fig. 2, E and F). Although C18orf1 can make a heteromeric complex with TMEPAI, C18orf1 and TMEPAI could independently suppress TGF-β signaling. To investigate the possibility that C18orf1α1 and C18orf1α2 can inhibit Smad2 phosphorylation upon TGF-β receptor activation in a manner analogous to TMEPAI, Smad2 and constitutively active TGF-β type I receptor (termed ALK5ca) were transfected into COS7 cells with and without the C18orf1α subfamily. Expectedly, both C18orf1α1 and C18orf1α2...
could interfere with ALK5ca-mediated Smad2 phosphorylation in almost the same manner as TMEPAI (Fig. 2G). Because both C18ORF1α subfamilies show comparably inhibitory action on TGF-β signaling, we used the longest form of C18ORF1, C18ORF1α1, as a representative of the C18ORF1 family in the subsequent experiments. TGF-β promotes Smad3 phosphorylation as well as Smad2 phosphorylation via its ALK5 activation (1–3). As seen in Fig. 2H, C18ORF1α1 could counteract ALK5ca-mediated Smad3 phosphorylation. In addition, ALK4ca-mediated Smad2 phosphorylation was inhibited by C18ORF1α1 as well (Fig. 2F). We further investigated whether C18ORF1 could perturb TGF-β-mediated or activin-mediated phosphorylation of endogenous AR-Smads in NMuMG cells. As expected, phosphorylation of AR-Smads upon both TGF-β and activin stimulation was suppressed by C18ORF1α1 in cells (Fig. 2F). However, C18ORF1α1 did not influence Smad1 phosphorylation mediated...
by constitutively active BMP type IB receptor (ALK6ca) (Fig. 2K).
Therefore, C18ORF1 specifically inhibits TGF-β/activin signaling but not BMP signaling.

Interaction of Smads with C18ORF1—C18ORF1 possesses
the SIM domain in its structure. Thus, AR-Smads possibly associate with C18ORF1. Fig. 3A indicates that AR-Smads can bind to C18ORF1α1, although none of the BMP-specific R-Smads (BR-Smads), Smad4, or Smad7 interact with C18ORF1α1. Indeed, the mutation of the SIM domain in C18ORF1 led C18ORF1α1 to lose its ability to associate with AR-Smads (Fig. 4, F and G). Next, we adopted PLA to detect endogenous interaction between AR-Smads and C18ORF1 in cells. When both rabbit anti-Smad3 and mouse anti-C18ORF1 antibodies were simultaneously added to the fixed sample, a large number of red spots could be observed (Fig. 3B). Conversely, no dots were detected when mouse anti-Myc9E10 and rabbit anti-Smad3 antibodies were used for PLA (Fig. 3C). This evidence indicates that C18ORF1 could associate with Smad3 in the cytosol.

Although C18ORF1 could interact with both Smad2 and Smad3, we did not know whether phosphorylation of AR-Smads at their C terminus affects the interaction between C18ORF1 and AR-Smads. Thus, we prepared lysates from cells transfected with either AR-Smad (FLAG-Smad2 or FLAG-Smad3) alone or with AR-Smad and ALK5ca/HA. Subsequently, each lysate was mixed with lysate prepared from cells transfected with C18ORF1α1/V5 alone, immunoprecipitated with anti-FLAG antibody, and then analyzed by Western blot with anti-V5 antibody. As seen in Fig. 3D, C18ORF1α1 bound to both nonphosphorylated and phosphorylated AR-Smads equally.

After TGF-β stimulation, phosphorylated AR-Smads interact with Smad4 to go to the nucleus, whereas this heteromeric complex transcriptionally regulates gene expression (1–3). Consistent with this concept, overexpression of C18ORF1α1 perturbed complex formation between AR-Smads and Smad4 upon TGF-β stimulation. (Fig. 3, E and F).

Pursuit of the Functional Domain in C18ORF1—C18ORF1 consists of a short extracellular domain, a transmembrane, and an intracellular domain. Thus, we examined which domain(s) in C18ORF1 is involved in its inhibition of TGF-β signaling. For that purpose, we first made a mutant that lacks both the extracellular domain and the transmembrane in C18ORF1α1 (C18ORF1α1ATM) (Fig. 4A). C18ORF1α1ATM possessed the ability to inhibit TGF-β signaling comparable with that of wild-type C18ORF1α1 (Fig. 4B). Because C18ORF1α1ATM can localize to cytosol, it might bind to AR-Smads to interfere with TGF-β signaling (Fig. 4C). The intracellular domain in C18ORF1 includes three known regions as follows: two PY motifs and one SIM domain. The deletion of two PY motifs from C18ORF1α1 (C18ORF1α1ΔPY) led to partial loss of its ability to inhibit TGF-β-induced luciferase activity, whereas C18ORF1α1 (Fig. 4A), resulted in a defect in its inhibitory action (Fig. 4B). Furthermore, C18ORF1α1 (Fig. 4A) could not inhibit ALK5ca-mediated phosphorylation of AR-Smads, although C18ORF1α1ΔPY still possessed the ability to partially inhibit AR-Smads phosphorylation upon ALK5 activation (Fig. 4, D and E). We previously reported that the SIM domain in TMEPAI interacts with AR-Smads (13). Indeed, the mutation of the SIM domain in C18ORF1α1 prevented it from associating with AR-Smads (Fig. 4, F and G), suggesting that the SIM domain in C18ORF1 plays a key role in its inhibition of TGF-β signaling. Consistently, overexpression of C18ORF1α1 in 911 cells kept AR-Smads in the cytosol upon TGF-β stimulation, whereas C18ORF1α1 (Fig. 4A) did not affect the nuclear translocation of AR-Smads in the presence of TGF-β (Fig. 4H). As shown in Fig. 1E, both C18ORF1α1 and TMEPAI can indeed colocalize to early endosomes. This result led us to the notion that C18ORF1 can compete with SARA for binding to AR-Smads, like TMEPAI. We have already reported that the FYVE domain of SARA, termed SARA(FYVE), colocalizes with SARA in early endosomes (26). Therefore, C18ORF1α1/V5 or TMEPAI/V5 was transfected into 911 cells with GFP-SARA(FYVE) instead of SARA because of low expression of SARA in cells. Like TMEPAI and GFP-SARA(FYVE), both C18ORF1α1 and GFP-SARA(FYVE) showed punctate staining indicative of their colocalization (Fig. 4J). SARA is known to recruit AR-Smads to present them to activated ALK5 (27). Thus, C18ORF1 might counteract with SARA in recruitment of AR-Smads. Indeed, SARA(SBD) blocked the interaction between C18ORF1α1 and AR-Smads (Fig. 4, J and K). These results reveal that SARA-AR-Smad complex formation is abrogated by C18ORF1. Therefore, C18ORF1 cannot efficiently recruit AR-Smads to ALK5 upon TGF-β stimulation in the presence of C18ORF1.

Gain-of-Function Analysis of C18ORF1—p21 and JunB are well known direct target genes of TGF-β signaling (28, 29). When C18ORF1α1 was introduced into HaCaT cells by adenoviral transfer, the induction of p21 and JunB proteins was inhibited. Conversely, C18ORF1α1 (Fig. 4A) deficient of the ability

![FIGURE 5.](image-url)
Negative Regulator of TGF-β Signaling

FIGURE 6. Loss-of-function analysis of C18ORF1. A, a decreased expression of C18ORF1 mRNA. The expressions of C18ORF1 and β-actin mRNAs as a negative control are shown. RT(+), with reverse transcriptase; RT(−), without reverse transcriptase. Cont, control siRNA; C18, C18ORF1-specific siRNA. B, effect of C18ORF1 siRNA on TGF-β-induced luciferase reporter activity. C, proliferation of TGF-β-induced Smad2 phosphorylation by C18ORF1 siRNA. C18ORF1 or control siRNA was incorporated into cells. Next, the cells were divided into six dishes and then stimulated with TGF-β for the indicated times. The expressions of phosphorylated Smad2 (upper panel), Smad2 (middle panel), and β-actin (lower panel) were observed using each specific antibody. D, mouse C18orf1(mC18orf1) rescues suppression of TGF-β-mediated responses by human C18ORF1-specific siRNA. All values represent mean ± S.D. Significantly different from human C18ORF1-specific siRNA-transfected cells in the presence of TGF-β *p < 0.05; **p < 0.001. E, enhancement of TGF-β-induced p21 expression by C18ORF1 siRNA. C18ORF1 or control siRNA was transfected into cells. Next, the cells were separated into two dishes, and cells in one dish were stimulated with TGF-β for 4 h. Upper panel, expression of C18ORF1 by RT-PCR; 2nd panel, expressions of phosphorylated Smad2; 3rd panel, expression of p21; lower panel, expression of β-actin. F, enhancement of TGF-β-induced TMEPAI and JUNB expression by C18ORF1 siRNA. C18ORF1 or control siRNA was transfected into cells. Next, the cells were separated into four dishes, and the cells in one dish were stimulated with TGF-β for the indicated times. The expression of each molecule was detected by RT-PCR. Upper panel, expression of C18ORF1; 2nd panel, expression of TMEPAI; 3rd panel, expression of JunB; lower panel, expression of β-actin. WB, Western blot.

to bind to AR-Smads, could not interfere with TGF-β-induced expression of p21 and JunB (Fig. 5).

Loss-of-Function Analysis of C18ORF1—Because overexpression of C18ORF1 blocks TGF-β signaling, decreased expression of C18ORF1 (Fig. 6A) might enhance it. The siRNA-mediated depletion of C18ORF1 could enhance both (CAGA)$_{12}$-luc reporter activity (Fig. 6B) and Smad2 phosphorylation (Fig. 6C) upon TGF-β stimulation. Furthermore, introduction of mouse C18orf1 could rescue suppression of TGF-β-induced (CAGA)$_{12}$-luc reporter activity by human C18ORF1 siRNA (Fig. 6D). Consistent with prolongation of Smad2 phosphorylation by TGF-β, siRNAs for C18ORF1 potentiated the TGF-β-induced expression of JUNB, p21, and TMEPAI more than did control siRNAs (Fig. 6, E and F).

TGF-β is known to promote cell motility in some epithelial cells. Thus, we tried to decrease the endogenous C18ORF1 expression using a lentiviral shRNA to explore the effect of C18ORF1 on cell motility. As seen in Fig. 7A, both C18ORF1-specific shRNAs could decrease the expression of C18ORF1 mRNA. Then, we tested the effect of TGF-β on cell migration using A549 cells expressing control shRNA, C18ORF1 shRNA#1, or shRNA#2. A549 cells expressing C18ORF1 shRNA#1 or shRNA#2 could migrate quicker than those expressing control shRNA (Fig. 7B and supplemental movie). Furthermore, we compared the ability of TGF-β-induced epithelial-mesenchymal transition (EMT) among the three cell lines. EMT is a process whereby tightly interacting and immotile epithelial cells acquire the phenotype of loosely adherent and motile mesenchymal cells. Consistent with the results of the cell migration assay, introduction of C18ORF1 shRNA#1 or C18ORF1 shRNA#2 promoted EMT in A549 cells more rapidly than did introduction of control shRNA when cells were stimulated with TGF-β (Fig. 7C). During EMT, E-cadherin, a marker of epithelial cells, gradually disappears. TGF-β prompted loss of E-cadherin expression in both shRNA#1- and shRNA#2-expressing A549 cells more quickly than in control shRNA-expressing A549 cells (Fig. 7, D and E). These gain-of-function and loss-of-function analyses of C18ORF1 provided a convincing explanation for how C18ORF1 interferes with TGF-β signaling.
DISCUSSION

Dysregulation of signal transduction promotes incompetent cell-cell communication to give rise to congenital or acquired disease. Thus, cells constantly survey inappropriate signal transductions to eliminate them. The TGF-β/H9252 family controls proliferation and differentiation in a variety of cell types. Accordingly, this signaling pathway is implicated in homeostasis during embryogenesis and in adult life. Not surprisingly, aberrant TGF-β/H9252 family signal transduction is linked to genetic disorders, tumorigenicity, and fibrosis, which can all be life-threatening (9, 10).

Because TMEPAI, a direct target gene of TGF-β signaling, contributes to a negative feedback loop of TGF-β signaling (13), we speculated that there is a TMEPAI homologue(s) that monitors excessive TGF-β or BMP signaling in cells. Our search of the NCBI database yielded C18ORF1 as a homologue of TMEPAI. Although the putative extracellular domain of C18ORF1 was less similar to that of TMEPAI, the putative intracellular domain between C18ORF1 and TMEPAI was highly conserved. As anticipated, TGF-β signaling, but not BMP signaling, could be counteracted by C18ORF1, as it is by TMEPAI.

We found six alternative splicing forms of C18ORF1 in the database. On the basis of their similarity, the six isoforms were classified into three C18ORF1 subfamilies as follows: C18ORF1α, C18ORF1β, and C18ORF1γ. Each subfamily shares the same intracellular domain except for a region consisting of 18 amino acids, whereas their N-terminal extracellular domains are divergent because of different exon usages. Among the six C18ORF1 isoforms, we focused on the C18ORF1β subfamily because RT-PCR supported the notion that C18ORF1α and C18ORF1β lacking the region consisting of 18 amino acids in its intracellular domain might be more highly expressed than others, although we did not know exactly how much the C18ORF1α subfamily is expressed in cells as compared with the C18ORF1β and C18ORF1γ subfamilies. When we sought to clarify how important the region, consisting of 18 amino acids in its intracellular domain, is for the ability of C18ORF1α to function, we could not find any differences among the C18ORF1α subfamily members. Because the region consisting of 18 amino acids is located apart from the SIM domain, the deletion of this region possibly does not affect the inhibitory action of C18ORF1 on TGF-β signaling. Furthermore, the extracellular domain of C18ORF1α is not necessary for...
C18ORF1α1 to reveal its inhibitory action for TGF-β signaling. Thus, C18ORF1β and C18ORF1γ subfamilies are strongly suggested to possess the inhibitory capability like the C18ORF1α subfamily because their intracellular domains are identical. Therefore, we used C18ORF1α1 in almost of all of the experiments in this study.

C18ORF1 colocalizes with both TMEPAI and SARA, revealing that C18ORF1 is present in early endosomes together with TMEPAI. Indeed, C18ORF1 could make a heteromeric complex with TMEPAI and C18ORF1 in mouse embryonic fibroblasts from C18orf1 and Tmepai knock-out mice, respectively (Fig. 2, E and F). Furthermore, inhibition of TGF-β signaling could not be synergistically seen when increased amounts of TMEPAI and C18ORF1α1 expression vectors were transfected in cells carrying a definite quantity of C18ORF1α1 and TMEPAI, respectively (Fig. 2, C and D). These results supposed that the heteromeric complex might not be needed for the function of C18ORF1 and TMEPAI.

SARA, which possesses a FYVE domain in its structure, has been demonstrated to present AR-Smads to activated ALK5 via its SBD. Thus, SARA seems to be a scaffold protein in TGF-β signaling (27). In this study, C18ORF1 could compete with SARA for binding to AR-Smads. Furthermore, SARA could improve the C18ORF1-mediated (this study) and TMEPAI-mediated (13) inhibition of TGF-β signaling. However, Bakkebo et al. (30) argued that SARA contributes to TGF-β signaling because a loss-of-function analysis of SARA did not affect TGF-β signaling at all. Because several scaffold molecules rely on activated ALK5 and AR-Smads (27, 31, 32), requirement of SARA for TGF-β signaling might be context-dependent. Therefore, C18ORF1 as well as TMEPAI possibly acts as a negative regulator of TGF-β signaling in cells that command SARA or its related molecules to activate the TGF-β signal.

We initially thought that C18ORF1 can ubiquitinate Smads via two PY motifs with which WW domain-containing E3 ligases interact. In fact, Smurfs, WWP1, and NEDD4 could be bound by C18ORF1 via its two PY motifs. However, we could not obtain any obvious evidence that Smads were either ubiquitinated or degraded.

When we tried to account for the role of the PY motifs and SIM domain in C18ORF1, C18ORF1ΔPY partially inhibited TGF-β signaling, in contrast to TMEPAIΔPY (13), although C18ORF1(4A) had a loss of its function. Deletion of the two PY motifs in C18ORF1 could affect the binding of its SIM domain to AR-Smads, or the WW domain-containing proteins, other than ubiquitin E3 ligases, could be involved in the inhibitory action of TGF-β signaling via the PY motifs of C18ORF1. We will explore the latter possibility in future investigations.

The loss-of-function and gain-of-function analyses indicated that C18ORF1 definitely interferes with the TGF-β signaling

FIGURE 8. Proposed model for TMEPAI family to inhibit TGF-β signaling. Both C18ORF1 and TMEPAI belong to TMEPAI family. C18ORF1 perturbs TGF-β signaling under steady state, whereas TMEPAI, which is a direct target gene of TGF-β signaling, is not expressed under steady state. However, as soon as cells receive TGF-β signaling, TMEPAI is immediately induced to be able to protect cells from excessive TGF-β signaling together with C18ORF1. In consequence, such extraordinary TGF-β signaling passes away. TMEPAI family makes either a heteromeric or homomeric complex even though heteromeric complex formation is of little importance for their function.
pathway. Therefore, it seems that the expression of TGF-β target genes and TGF-β-mediated cell motility were perturbed by C18ORF1, as they are by TMEPAI. The question then arises as to why two molecules that possess similar features to inhibit TGF-β signaling exist in one cell. It is known that most cells secrete TGF-β to communicate with neighboring cells, thereby ensuring maintenance of homeostasis. We speculate that C18ORF1 plays a role in fine-tuning TGF-β signaling under the steady state. However, TMEPAI, which is rapidly induced by TGF-β (13, 24), can protect cells from excessive TGF-β signaling together with C18ORF1 to cease such extraordinary TGF-β signaling (Fig. 8). Although it is well known that a large number of inhibitory molecules for TGF-β signaling are present in cells (11, 12), so far only TMEPAI is known to be an inhibitory molecule of TGF-β signaling that targets SARA (13). In this study, we demonstrated that C18ORF1 coordinates with TMEPAI to target SARA for interference of TGF-β signaling.

TGF-β has two aspects during tumorigenicity as follows: tumor-suppressive effects in the premalignant state and tumor progression in the malignant state (33–36). TMEPAI is known to be expressed in several tumors (13, 24, 37–41). Thus, TMEPAI seems to have tumor-promoting effects due to interference of TGF-β-mediated growth inhibition. However, no relevance between C18ORF1 and tumorigenicity has yet been reported. Genetic mouse model and tumor studies are needed to clarify the implication of C18ORF1 in tumorigenicity. However, C18ORF1 loci confer susceptibility to schizophrenia (14–16). Forebrain-specific Smad4 knock-out mice revealed psychiatric-like behavior because of a disruption in the balance of the excitatory and inhibitory hippocampal networks. Thus, these Smad4 conditional mice show an endophenotype of schizophrenia (42). Therefore, deletion of C18ORF1 that is constitutively expressed in cells might also disqualify the excitatory and inhibitory hippocampal networks, leading to development of schizophrenia.

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