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# *Smad7*-deficient mice show growth retardation with reduced viability

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**Smad7 is an inhibitory molecule induced by members of the transforming growth factor-β (TGF-β) family, including TGF-β, activin, nodal and bone morphogenetic proteins (BMPs). To elucidate the *in vivo* functions of Smad7, we generated conditional *Smad7*-knockout mice in which the Mad homology 2 (MH2) domain and the poly (A) signal sequence were flanked with loxP sites (floxed). The *Smad7*-floxed mice exhibited no obvious phenotype. *Smad7* total-null mice on a C57BL/6 background died within a few days of birth, whereas mice with an ICR background developed to adulthood but were significantly smaller than wild-type mice. Unexpectedly, phospho-Smad2 and phospho-Smad3 were decreased in *Smad7*-deficient mouse embryonic fibroblast (MEF) cells, whereas phospho-Smad1/5/8 was similarly expressed in wild-type and *Smad7*-deficient MEF cells. Moreover, expression levels of TGF-β type I receptor (ALK5) were higher in *Smad7*-deficient MEF cells than in wild-type MEF cells. Plasminogen activator inhibitor-1 (PAI-1) and inhibitor of differentiation-1 (Id-1) mRNA were similarly expressed in wild-type and *Smad7*-deficient MEF cells. Some differences were observed in mitogen-activated protein kinase (MAPK)-signalling between wild-type and *Smad7*-deficient MEF cells. We demonstrated that Smad7 plays an important role in normal mouse growth and provide a useful tool for analysing Smad7 functions *in vivo*.**

*Keywords*: developmental factors/growth factors/knockout/mouse/protein kinases.

*Abbreviations*: ALK5, Activin receptor-like kinase-5; BMP, bone morphogenetic protein; BrdU, bromo-deoxyuridine; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MH2, Mad homology 2; PCR, polymerase chain reaction; TGF-β, transforming growth factor-β.

Members of the transforming growth factor-β (TGF-β) family, including TGF-β, activin, nodal and bone morphogenetic proteins (BMPs), are multifunctional proteins with a broad spectrum of cellular activities, including cell proliferation, differentiation, adhesion, migration and apoptosis (*1*,*2*). Members of the TGF-β family transduce signals through two distinct serine/threonine kinase receptors, types I and II (*3*–*5*). After ligand binding to the receptors, Smad family proteins mediate intracellular signalling.

Smad proteins in mammals consist of eight subtypes and are classified into three groups: receptor-regulated Smads (R-Smads), common-partner Smad (Co-Smad) and inhibitory Smads (I-Smads). Smad2 and 3 are R-Smads, which are activated by TGF-β, activin and nodal, whereas Smad1, 5 and 8 are BMP-specific R-Smads (*6*). The C-terminus of R-Smads contains a Ser-Ser-X-Ser motif phosphorylated by type I receptors. Phosphorylated R-Smads form complexes with Co-Smad (Smad4) and translocate into the nucleus, where they bind to the regulatory regions of target genes. Smad6 and 7 are I-Smads, and they inhibit TGF-β family signalling through multiple mechanisms, including preventing the activation of R-Smads by competing for binding to activated type I receptors (*7*–*10*). I-Smads contain a conserved C-terminal region termed the Mad homology 2 (MH2) domain, which is essential for inhibiting TGF-β family signalling (*11*). Smad6 preferentially suppresses BMP signalling activated by certain BMP type I receptors, including ALK3 and ALK6, while Smad7 inhibits both BMP and TGF-β signalling (*11*–*14*). Since the expression of I-Smads is induced by TGF-β family proteins, they form an inhibitory feedback loop to control the amplitude or duration of TGF-β-Smad signalling (*15*–*17*). Smad7 also acts as an adaptor protein that recruits Smurf1/2 to the TGF-β receptor complex to promote its degradation (*18*, *19*).

There are many reports on close interactions between Smad and mitogen-activated protein kinase (MAPK) signalling (*20*). Smad7 is reported to function

as a crosstalk factor. In pre-chondrogenic cells, Smad7 suppresses chondrocytic differentiation by decreasing phosphorylation of p38 (*21*). TGF-β induces apoptosis by activating p38 MAPK in prostate cancer cells, in which Smad7 acts as a scaffold protein (*22*, *23*). Smad7 also activates JNK signalling and induces apoptosis in certain cell types (*24*). Thus, Smad7 plays a complex role in a context-dependent manner.

Smad7 plays a critical role in various physiological processes, and abnormal expression of Smad7 has been reported in patients with scleroderma and inflammatory bowel disease (*25*, *26*). Some groups have reported *in vivo* functions of Smad7 by using knockout mice (*27–29*). However, deleting exon 1 of the *Smad7* gene resulted in the expression of a truncated Smad7 protein that included the MH2 domain (*27*). Although other investigators flanked only the MH2 domain of *Smad7* with loxP sites, they did not target the entire sequence of exon 4, which contained the MH2 domain and the poly (A) signal sequence (*28*). This targeting strategy may lead to the expression of a truncated Smad7 protein, owing to the presence of the mRNA-stabilizing poly (A) signal sequence.

In the present study, we generated mice carrying a conditional *Smad7* allele in which the entire exon 4 sequences were flanked with loxP sites (floxed). Moreover, we introduced sequences containing a splice acceptor site of exon 4 as well as three stop codons behind the second loxP site. We expected that after Cre recombinase excised the *Smad7*-floxed allele, any transcripts from exons 1 to 3 could bind to three stop codons and degrade these transcripts. To analyse the phenotype of *Smad7*-deficient mice, we crossed these mice with Ayu-1-Cre transgenic mice, which ubiquitously expressed Cre recombinase (*30*). *Smad7*-deficient mice on a C57BL/6 background showed a perinatal lethal phenotype, while mice on an ICR background survived to adulthood but showed significantly reduced body weight. *Smad7*-floxed mice were viable, fertile and indistinguishable from wild-type mice. We also analysed the phenotypes of mouse embryonic fibroblast (MEF) cells.

## Materials and Methods

### *Generation of* Smad7 *-floxed and* Smad7 *-knockout mice*

We used a mouse bacterial artificial chromosome (BAC) clone RP23-24N20 (Interbiotechno; Tokyo, Japan) to prepare *Smad7* gene fragments. To disrupt both the MH2 domain and the poly (A) signal sequence of the *Smad7* gene, we designed a targeting vector in which the entire sequence of exon 4 was flanked by two loxP sites. The first loxP site was introduced ~1.2-kb upstream of exon 4 and the second loxP site was introduced ~150-bp downstream of exon 4. The second loxP site was followed by a 1.2-kb intron sequence, a splice acceptor site of exon 4, 17 bp of exon 4 lacking the MH2 domain and three stop codons. Neomycin-resistant gene cassettes [mc1-neo-poly (A)] flanked by flippase recognition target (FRT) sites were ligated next to the three stop codons for use in positive selection. The diphtheria toxin (*DT-A*) gene was attached to 3′-end of the targeting vector for use in negative selection (Fig. 1A). We electroporated the linearized targeting vector into mouse E14 embryonic stem (ES) cells and selected G418-resistant clones. Homologous recombinants were screened using polymerase chain reaction (PCR) and Southern blot analyses. The PCR primers used were as follows: 5′-CATACCACCCCGAGG ACACTGTCA-3′ and 5′-GATGCAATGCGGCGGCTGCATACG CTTG-3′. Southern blot analysis was performed by the digestion of genomic DNA using *Apa*I, hybridization to specific probes, and detection using the digoxigenin luminescent detection kit (Roche; Basel, Switzerland) according to the manufacturer's instructions. The probes were amplified using the following primers: 5′-AGCG AATGGATTGTGGGGGATG-3′ and 5′-ACCTCAGAGATTCCA AAGTGGACCCAG-3′. Chimeric mice were generated by injecting targeted ES cells into C57BL/6 blastocysts. To obtain mice heterozygous for the *Smad7*-floxed allele (*Smad7* $^{F/+}$ mice), chimeric males were mated with C57BL/6 females. Genotyping of mice was performed using PCR with the following three primers: 5′-TTCAGA GGCAGACCGAACCTCCAA-3′, 5′-AGGATTGGGTCAGGGA CAGAAGAGCA-3′ and 5′-TCTCACCTTGCTCCTGCCGAGAA AGTA-3′. Sizes of the wild-type and mutant fragments were 897 and 639 bp, respectively. To obtain mice heterozygous for the *Smad7*-deficient allele (*Smad7*$^{+/-}$ mice), *Smad7*$^{F/+}$ mice were crossed with Ayu-1-Cre transgenic mice, which broadly express Cre recombinase, including expression in germ cells (*30*). Ayu-1-Cre transgenic mice on a C57BL/6 and ICR background were used, and the resultant B6-*Smad7*$^{+/-}$ and ICR-*Smad7*$^{+/-}$ mice were intercrossed to produce *Smad7*-knockout mice (*Smad7*$^{-/-}$ mice) in both genetic backgrounds. B6-*Smad7*$^{+/-}$ and B6- *Smad7*$^{F/+}$ mice have been backcrossed onto a C57BL/6 background for more than six generations. All mice were housed in specific pathogen-free facilities and treated under the experimental protocol approved by the Ethics Review Committee for Animal Experimentation of Tsukuba University.

### *Cell culture and stimulation of MEF cells*

Primary MEF cells were isolated from embryonic Day 14.5 (E14.5) embryos with a C57BL/6 background. MEF cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 100 U/ml penicillin G, 2 mM L-glutamine, 100 μM non-essential amino acids, 100 μg/ml streptomycin, 100 μM 2-mercaptoethanol and 1 mM sodium pyruvate. To detect endogenous phosphorylated R-Smads or Smad7, MEF cells were serum-starved overnight and then treated using 1.5 ng/ml of TGF-β1 or 20 ng/ml of BMP4 for the indicated periods of time. For the expression of phospho-p38 (p-p38) and phospho-JNK (p-JNK), serum-starved MEF cells were treated using 10 ng/ml TGF-β1, 500 μM $H_2O_2$, or 500 mM NaCl for the indicated periods of time. TGF-β1 and BMP-4 were purchased from PeproTech (Rocky Hill, NJ, USA) and R&D Systems (Minneapolis, MN, USA), respectively.

### *Immunoblotting*

Cell extracts were prepared using Nonidet P-40 lysis buffer containing 20 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.5% NP-40 and 1 mM phenylmethylsulphonyl fluoride, or a lysis buffer containing 25 mM NaF and 1 mM $Na_3VO_4$. Protein concentrations of supernatants were measured, and equal amounts of total proteins were separated using 8.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by wet transfer of proteins to a Pall Fluorotrans membrane (Pall Life Sciences; Port Washington, NY, USA). The following antibodies were used: anti-Smad7 (N19), anti-ALK5 (V-22) (Santa Cruz Biotechnology; Santa Cruz, CA, USA), anti-Smad2/3 (BD Transduction Laboratories; Lexington, KY, USA), anti-phospho-Smad2, anti-phospho-Smad3, anti-phospho-Smad1/5/8, anti-phospho-p38, anti-phospho-JNK (Cell signalling; Danvers, MA, USA) and anti-tubulin (DM 1 A) (Sigma; St Louis, MO, USA). Intensities of immunoblot bands were quantified using ImageJ software (National Institutes of Health; Bethesda, MD, USA), and the values were normalized to the loading control for each lane.

### *Quantitative real-time reverse transcription–PCR*

Total RNAs were extracted using Isogen reagent (Nippon Gene; Tokyo, Japan). First-strand cDNAs were synthesized using oligo (dT) primers and the SuperScript First-Strand Synthesis System (Invitrogen; Carlsbad, CA, USA). Quantitative real-time reverse transcription–PCR (RT–PCR) was performed as described previously (*31*). Primer sequences were as follows: *Smad7*, 5′-TTCGGA CAACAAGAGTCAGCTGGT-3′ and 5′-AGCCTTGATGGAGA AACCAGGGAA-3′; hypoxanthine phosphoribosyltransferase 1 (HPRT1): 5′-CTGGTTAAGCAGTACAGCCCCA-3′ and 5′-GGT CCTTTTCACCAGCAAGCT-3′; plasminogen activator inhibitor-1 (PAI-1): 5′-CCACAAAGGTCTCATGGACCAT-3′ and 5′-TGAA

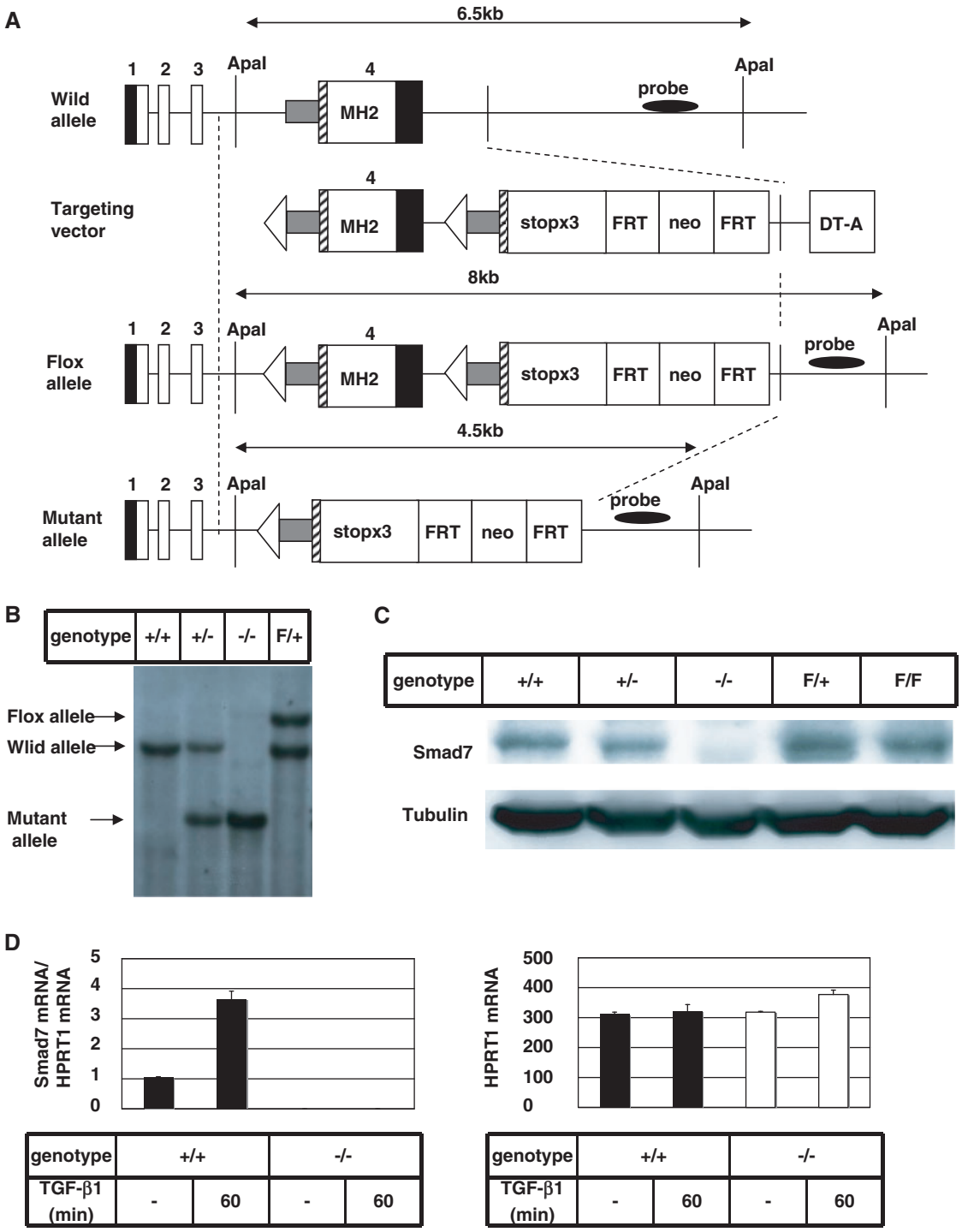


**Fig. 1 Generation of *Smad7*-floxed and *Smad7*-knockout mice.** (A) Schematic representation of the exon-intron structure of the *Smad7* gene and the outcome of homologous recombination. White and black boxes indicate coding exons and non-coding exons, respectively. Gray boxes indicate genomic sequences ~1.2-kb upstream of exon 4. Diagonal-striped boxes represent the first 17 bp in the exon 4. The probe used for Southern blot analysis is shown as a black oval. Open triangles denote the loxP sequence. MH2, MH2 domain; stopx3, three stop codons; neo, FRT-flanked neomycin-resistant gene cassette; DT-A, diphtheria toxin gene. (B) Southern blot analysis of genomic DNA from each genotype on an ICR background. Genomic DNA was extracted from mice tails, digested with *Apa*I and hybridized to the probe as shown in (A). Wild-type, floxed and mutant alleles are detected as 6.5-, 8- and 4.5-kb bands, respectively. (C) Immunoblotting of Smad7 and tubulin in MEF cells from mice of each genotype on a C57BL/6 background. MEF cells were serum-starved overnight and then treated with 1.5 ng/ml TGF-β1 for 3 h. Tubulin was used as a loading control. (D) Quantitative real time RT–PCR analyses of Smad7 and HPRT1 in MEF cells from each genotype mice on a C57BL/6 background. MEF cells were serum-starved overnight and then treated with or without 1.5 ng/ml TGF-β1 for 60 min. Each value of Smad7 expression was normalized to HPRT1 expression. Columns, mean; bars, SD.

AGTGTTGTGCCCTCCAC-3′; inhibitor of differentiation-1 (Id-1): 5′-AGGTGAACGTCCTGCTCTACGA-3′ and 5′-CAGGATCTCC ACCTTGCTCACT-3′. All samples were run in duplicate for each experiment. Expression values were normalized to the levels of HPRT1.

### *Statistics*

Statistical significance of mouse weight was evaluated using non-repeated analysis of variance (ANOVA). $^{\#}P<0.05$; $^{*}P<0.01$.

## Results

### *Generation of* Smad7 *-floxed and* Smad7 *-knockout mice*

The *Smad7* gene contains 4 exons; exon 4 includes the MH2 domain and poly (A) signal sequence. To delete the entire exon 4 using the Cre/loxP system, a targeting strategy was designed to flank the entire sequence of exon 4 by two loxP sites (Fig. 1A). *Smad7*$^{F/+}$ or *Smad7*$^{F/F}$ mice, which were expected to express intact Smad7, were viable, healthy and fertile. To generate *Smad7*-null mice, we crossed *Smad7*$^{F/+}$ mice with Ayu-1-Cre transgenic mice that broadly express Cre recombinase, including expression in germ cells (*30*). *Smad7*$^{F/+}$ mice were mated with transgenic mice lines on an ICR and C57BL/6 background. ICR-*Smad7*$^{+/-}$ and B6-*Smad7*$^{+/-}$ mice were obtained and then intercrossed to produce ICR-*Smad7*$^{-/-}$ and B6-*Smad7*$^{-/-}$ mice, respectively. Southern blot analyses of genomic DNA confirmed that the *Smad7* gene was correctly targeted and excised by Cre recombinase (Fig. 1B). Immunoblots of Smad7 showed that Smad7 protein was expressed at wild-type levels in *Smad7*$^{F/+}$ and *Smad7*$^{F/F}$ MEF cells, whereas expression was reduced by approximately half in *Smad7*$^{+/-}$ and absent in *Smad7*$^{-/-}$ MEF cells (Fig. 1C). We also examined mRNA levels of *Smad7* by using quantitative real-time RT–PCR for MEF cells. TGF-β treatment of wild-type MEF cells led to induction of *Smad7* mRNA to ~4-fold. However, neither basal expression nor up-regulation of *Smad7* transcripts was detected in *Smad7*$^{-/-}$ MEF cells (Fig. 1D). These data demonstrate that *Smad7*-floxed allele functions normally and that its excision by Cre recombinase leads to null expression.

### *Survival and growth rate of* Smad7 *-knockout mice*

B6-*Smad7*$^{+/-}$ mice were intercrossed to produce B6-*Smad7*$^{-/-}$ mice. Table I shows the results obtained from mice backcrossed for more than six generations. B6-*Smad7*$^{-/-}$ mice were born at the expected Mendelian ratio, but many of them died before weaning (Table I).

In contrast to the severe phenotype of B6-*Smad7*$^{-/-}$ mice, approximately half of the ICR-*Smad7*$^{-/-}$ mice survived to adulthood (Table II). Body size and weight of ICR-*Smad7*$^{-/-}$ mice were smaller than that of their control litter for both sexes (Fig. 2A and B). These differences were not observed between *Smad7*$^{+/+}$, *Smad7*$^{F/+}$ and *Smad7*$^{F/F}$ mice (Fig. 2C), suggesting that the dwarfism phenotype is due to a loss of Smad7 expression.

**Table I. Viability of offspring from *Smad7* heterozygous intercrosses on a C57BL/6 background.**

| Age | +/+ | +/− | −/− | Total |
|---|---|---|---|---|
| E14.5 | 9 | 15 | 5 | 29 |
| P0 | 15 | 32 | 17 (6) | 64 (6) |
| P1 | 14 | 26 | 15 (7) | 55 (7) |
| P2-7 | 17 | 39 | 0 (5) | 61 (5) |

Genotype analysis of embryos at E14.5 and pups at post natal Day 0 (P0), P1 and P2-7. Numbers in parentheses refer to the number of dead embryos that could be genotyped.

**Table II. Viability of offspring from *Smad7* heterozygous intercrosses on an ICR background.**

| Age | +/+ | +/− | −/− | Total |
|---|---|---|---|---|
| > 3 weeks | 29 | 69 | 12 | 110 |

Genotype analysis of mice >3 weeks old.

### *Effects of* Smad7 *deletion on TGF-β or BMP signalling*

Because Smad7 is a major negative regulator of TGF-β and BMP signalling, loss of *Smad7* is expected to result in enhancement of these signalling activities. Therefore, we analysed the phosphorylation status of Smad2 and Smad3, which are downstream signalling molecules of TGF-β, and that of Smad1/5/8, which are downstream signalling molecules of BMP. We stimulated MEF cells using TGF-β1 or BMP4 and compared the profiles of phosphorylated Smads (p-Smads) on both short- and long-term scales. Unexpectedly, p-Smad2 and p-Smad3 were decreased in *Smad7*-deficient MEF cells at some time points in short-term scale (Fig. 3), whereas p-Smad1/5/8 was similarly expressed in wild-type and *Smad7*-deficient MEF cells (Fig. 4A–C). These findings suggest that the downstream signals of TGF-β but not BMP is down-regulated in *Smad7*$^{-/-}$ MEF cells. We also observed that the expression of TGF-β type I receptor (ALK5) was elevated in *Smad7*$^{-/-}$ MEF cells regardless of TGF-β treatment (Fig. 4D). These findings suggest that although ALK5 expression is up-regulated, the downstream signals of ALK5 are down-regulated by *Smad7*-deficiency.

We further analysed the expression levels of PAI-1 and Id-1, which were target genes of TGF-β and BMP respectively. We performed quantitative real-time RT–PCR using *Smad7*$^{+/+}$ and *Smad7*$^{-/-}$ MEF cells and found that there were no remarkable differences in the expression of PAI-1 and Id-1 between these MEF cells (Fig. 5). These results suggest that the intensity of TGF-β or BMP signalling is similar between *Smad7*$^{+/+}$ and *Smad7*$^{-/-}$ MEF cells.

### *Effects of* Smad7 *deletion on MAPK activation*

Many reports have shown that Smad7 acts as a cross-talk factor between Smad and MAPK signalling (*20–24*, *32*). Thus, we investigated the expression of p-p38 and p-JNK in *Smad7*$^{+/+}$ and *Smad7*$^{-/-}$ MEF cells stimulated by TGF-β. We found that the expression levels of p-p38 were decreased and those of

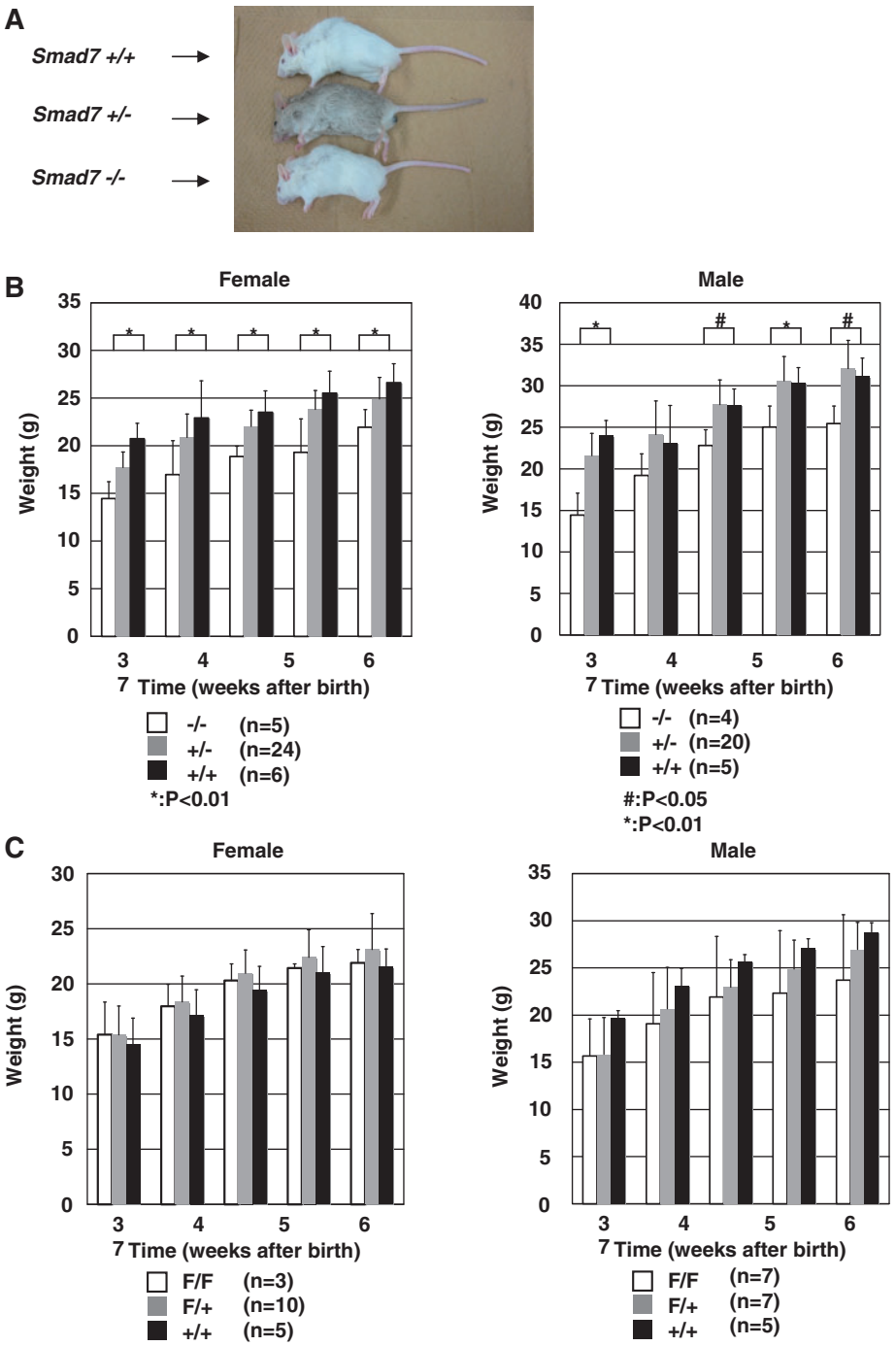


**Fig. 2** ***Smad7*-deficient mice on an ICR background were smaller than wild-type mice.** (A) Male body size in $Smad7^{+/+}$, $Smad7^{+/-}$ and $Smad7^{-/-}$ mice at the age of 12 weeks. (B) Body weight of $Smad7^{+/+}$, $Smad7^{+/-}$ and $Smad7^{-/-}$ mice. Male and female body weight in 3- to 7-week-old mice was measured. The number of mice is shown in parentheses. Data were analysed using non-repeated analysis of variance (ANOVA). Statistically significant reductions were observed among the indicated groups. Error bars represent standard deviation. $^{\#}P<0.05$; $^{*}P<0.01$. (C) Body weight of $Smad7^{+/+}$, $Smad7^{F/+}$ and $Smad7^{F/F}$ mice. There were no statistically significant differences among any groups.

p-JNK1 were slightly elevated in *Smad7*-deficient MEF cells, whereas those of p-JNK2/3 were not significantly different between $Smad7^{+/+}$ and $Smad7^{-/-}$ MEF cells (Fig. 6A). We also examined levels of p-p38 in response to oxidative or osmotic stresses; however, the results were nearly identical (Fig. 6B). These results indicate that Smad7 influences the MAPK activation pathway in response to TGF-β, but not other stresses.

## Discussion

Although Smad6 and Smad7 are both I-Smads, the functions of Smad7 are more extensive than those of Smad6 (*11*, *12*, *33*). Since *Smad6*-null mice showed severe defects in cardiovascular system development and homeostasis, *Smad7*-knockout mice were also expected to show a severe phenotype (*34*). To analyse

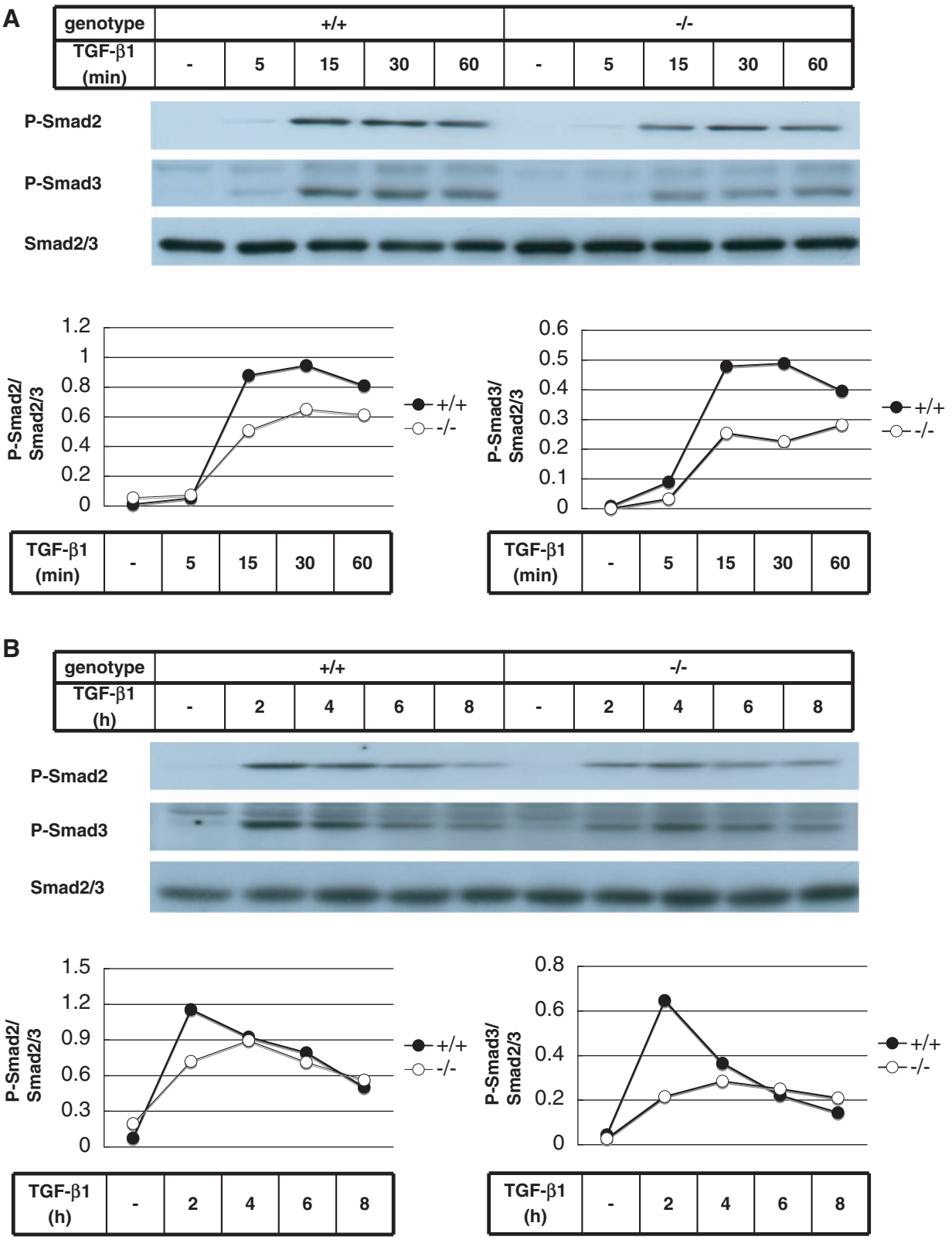


**Fig. 3 Phospho-Smad2/3 expression in *Smad7*-deficient MEF cells.** (A and B) Immunoblotting of phospho-Smad2/3 (p-Smad2/3) in *Smad7*$^{+/+}$ and *Smad7*$^{-/-}$ MEF cells. MEF cells were serum-starved overnight and then treated with 1.5 ng/ml TGF-β1 on both short- (A) and long- (B) term scales. Smad2/3 was blotted as a loading control. Phospho-Smad2/3 levels were measured and normalized by Smad2/3 using ImageJ software.

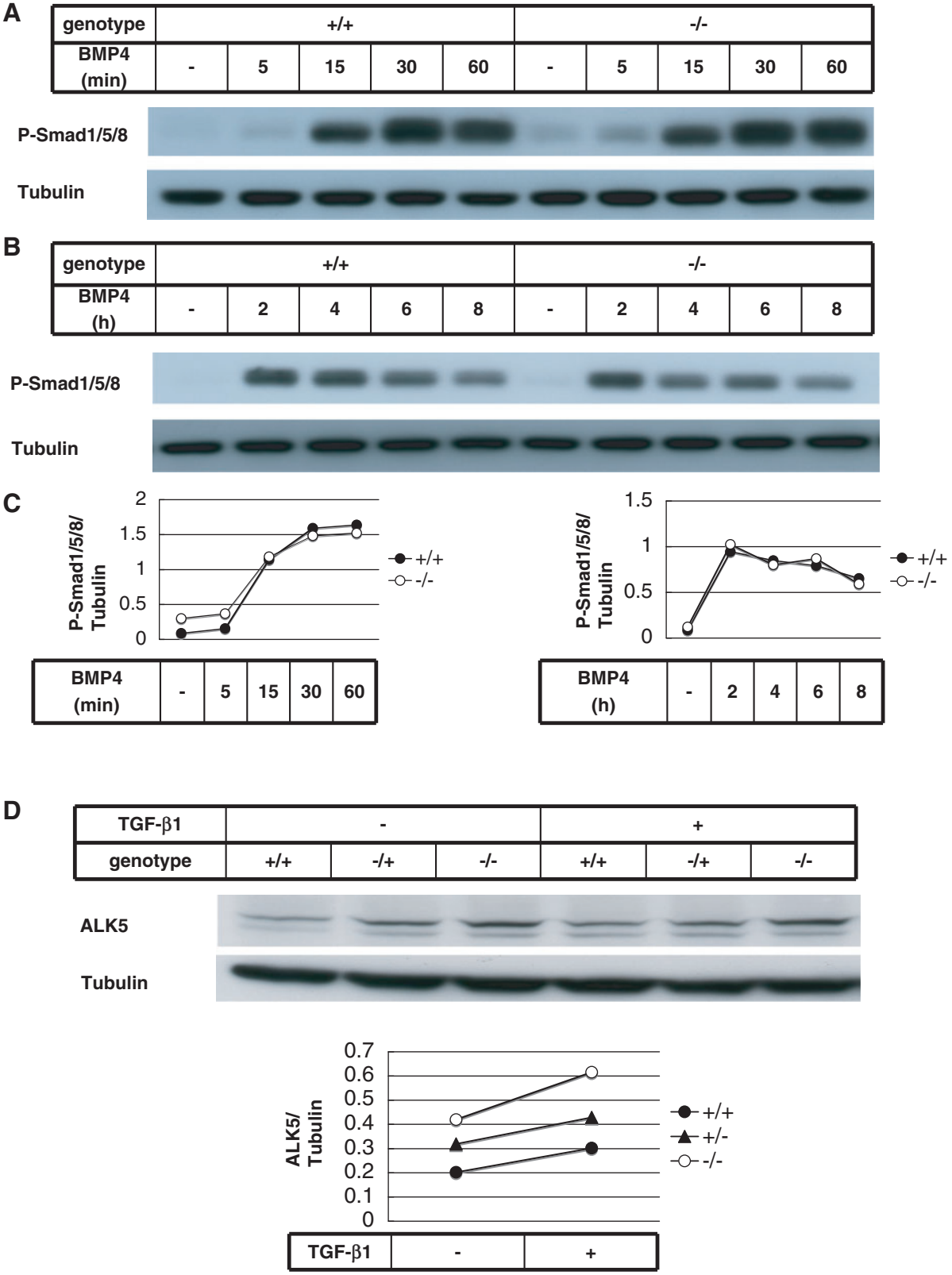


**Fig. 4 Phospho-Smad1/5/8 and ALK5 expression in *Smad7*-deficient MEF cells.** (A and B) Immunoblotting of phospho-Smad1/5/8 (p-Smad1/5/8) in *Smad7*$^{+/+}$ and *Smad7*$^{-/-}$ MEF cells. MEF cells were serum-starved overnight and then treated with 20 ng/ml BMP4 on both short- (A) and long- (B) term scales. Tubulin was used as a loading control. (C) Phospho-Smad1/5/8 levels of short- (left) and long- (right) term scales were normalized using ImageJ software. (D) Immunoblotting of ALK5 and tubulin in *Smad7*$^{+/+}$ and *Smad7*$^{-/-}$ MEF cells. MEF cells were serum-starved overnight and then treated with or without 1.5 ng/ml TGF-β1 for 30 min. ALK5 levels were measured and normalized by tubulin using ImageJ software.

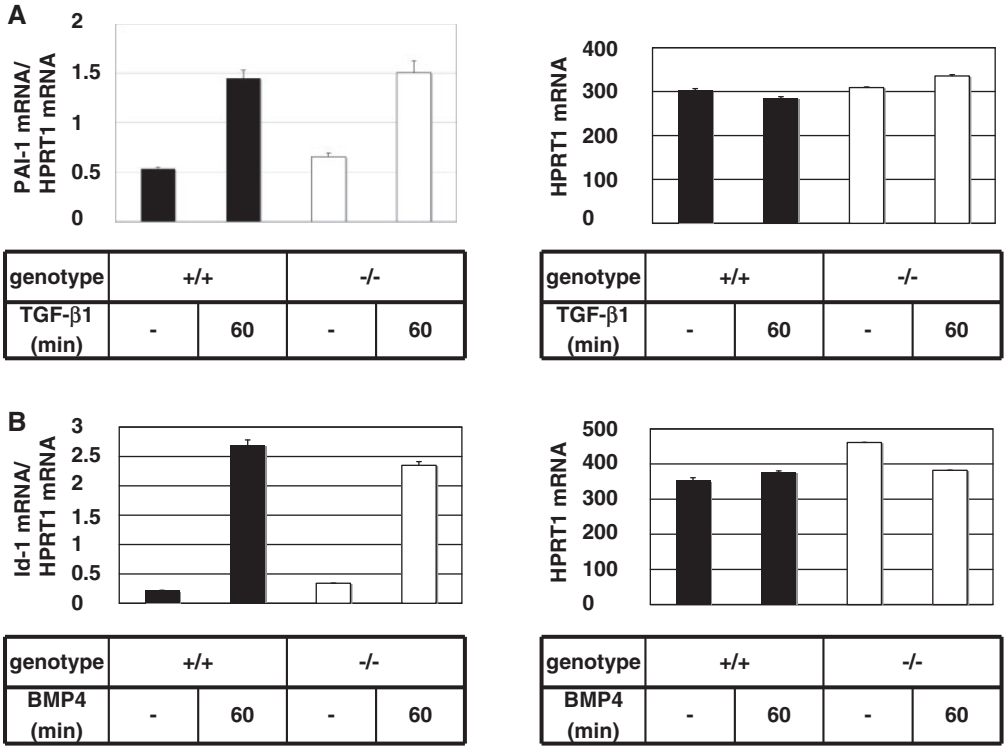


**Fig. 5 PAI-1 and Id-1 expression in *Smad7*-deficient MEF cells.** Quantitative real time RT–PCR analyses of PAI-1 (A) and Id-1 (B) in MEF cells from $Smad7^{+/+}$ and $Smad7^{-/-}$ MEF cells. MEF cells were serum-starved overnight and then treated with or without 1.5 ng/ml TGF-β1 (A) or 20 ng/ml BMP4 (B) for 60 min. Expression levels of PAI-1 and Id-1 were normalized by that of HPRT1. Columns, mean; bars, SD.

the *in vivo* functions of Smad7 under various conditions, we generated conditional knockout mice of *Smad7*. Exon 4 of the *Smad7* gene includes the MH2 domain and the poly (A) signal sequence. The former is important for the inhibitory function of Smad7 and the latter for Smad7 mRNA stability. Thus, we designed a construct to disrupt the MH2 domain and poly (A) signal sequence by inserting two loxP sites flanking exon 4. We also inserted sequences containing a splice acceptor site of exon 4 followed by three stop codons behind the second loxP site to prevent the production of an unpredictable aberrant fusion protein of the Smad7 N-terminus. $Smad7^{F/+}$ or $Smad7^{F/F}$ mice did not display an obvious phenotype and they expressed normal levels of *Smad7*. However, $Smad7^{-/-}$ MEF cells did not produce the *Smad7* transcript or express the Smad7 protein, suggesting that the *Smad7*-floxed allele functions normally and its excision by Cre recombinase successfully disrupted its function.

To explore the null phenotype of *Smad7* in mice, we crossed $Smad7^{F/+}$ mice with Ayu-1-Cre transgenic mice on ICR and C57BL/6 backgrounds. Approximately half of the ICR-$Smad7^{-/-}$ mice survived to adulthood and exhibited a dwarf phenotype, whereas most B6-$Smad7^{-/-}$ mice died before weaning. These results suggest that Smad7 is indispensable for normal growth of mice, and the severity of *Smad7* deficiency significantly differed according to genetic background. Phenotypes of TGF-β1 deficiency differ according to mouse strain (*35*). Thus, modifier genes that enhance or suppress the severity of the *Smad7*-deficient phenotype may exist. Future identification of such modifier genes may reveal novel regulatory mechanisms in TGF-β signalling. We also found that some B6-$Smad7^{-/-}$ mice died at post-natal Day 0. Since TGF-β family members have been reported to be expressed in the endometrium and placenta, these results suggest that *Smad7* deletion may lead to placental dysfunction (*36*).

We observed that ALK5 was increased in *Smad7*-deficient MEF cells. The up-regulation of ALK5 in *Smad7*-deficient cells may be due to impaired down-regulation of the TGF-β receptor complex by Smurf1/2. Nevertheless, the level of p-Smad2 and p-Smad3 were decreased and there were no marked differences between $Smad7^{+/+}$ and $Smad7^{-/-}$ MEF in the expression of PAI-1, which is a target gene of TGF-β. These results suggest that deletion of *Smad7* affects the expression levels of signal molecules of TGF-β, but does not influence the signal intensity of TGF-β under our experimental conditions. To limit the effects of *Smad7* deletion and keep the intensity levels of TGF-β, other factor(s) may have down-regulated p-Smads and compensated for the up-regulation of ALK5. Previous studies reported enhanced TGF-β signalling in T cells and the hearts of $Smad7^{-/-}$ mice (*27–29*). Therefore, we cannot exclude the possibility that deletion of *Smad7* influences TGF-β signalling in some organs.

MAPK interacts with TGF-β-signalling pathway in various situations (*20*), and Smad7 plays a complex role in a context-dependent manner (*20–24*, *32*). We found that the expression levels of p-p38 were

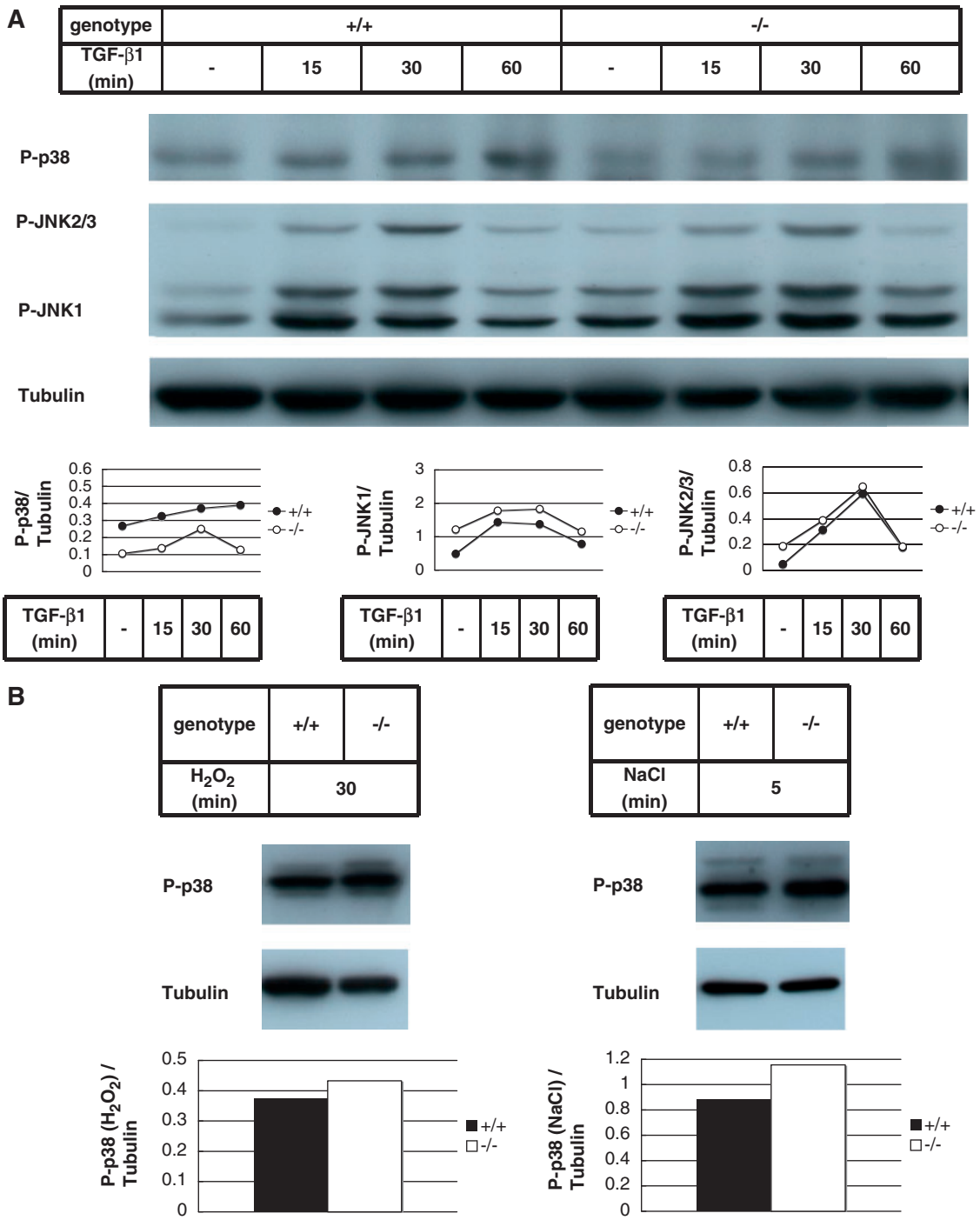


**Fig. 6 Phospho-p38 and phospho-JNK expression in *Smad7*-deficient MEF cells.** (A) Immunoblotting of phospho-p38 (p-p38) and phospho-JNK (p-JNK) in $Smad7^{+/+}$ and $Smad7^{-/-}$ MEF cells. MEF cells were serum-starved overnight and then treated with 10 ng/ml TGF-β1 for the indicated periods of time. Tubulin was used as a loading control. Phospho-p38 and phospho-JNK levels were normalized using ImageJ software. (B) Expression of phospho-p38 under oxidative or osmotic stress conditions in $Smad7^{+/+}$ and $Smad7^{-/-}$ MEF cells. MEF cells were serum-starved overnight and then treated with 500 μM $H_2O_2$ or 500 mM NaCl, respectively, for the indicated periods of time. Phospho-p38 levels under oxidative or osmotic stress conditions were normalized by tubulin using ImageJ software.

decreased and those of p-JNK1 were slightly elevated in *Smad7*-deficient MEF cells. The decrease of p-p38 level may be due to a direct effect of *Smad7* deficiency or by indirect effects such as the alterations in the expressions of p-Smads and ALK5. Further studies are required to elucidate the mechanism.

In conclusion, we report a mouse model for analysing Smad7 functions *in vivo*. By crossing $Smad7^{F/+}$ mice with various Cre transgenic mice, the *in vivo* functions of Smad7 can be elucidated in diverse situations, leading to the development of new therapies for Smad7-related diseases.

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### Conflict of interest

None declared.

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