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An ENU-induced p.C225S missense mutation in the mouse *Tgfb1* gene does not cause Camurati-Engelmann disease-like skeletal phenotypes

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Abstract: Camurati-Engelmann disease (CED) is a rare sclerosing bone disorder in humans with autosomal dominant inheritance. Mutations in the gene (*TGFB1*) that encodes transforming growth factor-β1 (TGF-β1) are causative for CED. TGF-β1 signaling is enhanced by the CED-causing mutations. In this study, we performed *Tgfb1* mutation screening in an ENU-mutagenized mouse genomic DNA library. We identified a missense mutation in which cysteine was substituted by serine at position 225 (p.C225S), that corresponded to the CED-causing mutation (p.C225R). TGF-β1 mutant protein carrying p.C225S was secreted normally into the extracellular space. Reporter gene assays showed that the p.C225S mutants enhanced TGF-β signaling at the same level as p.C225R mutants. We generated p.C225S homozygous mice and confirmed that the mature TGF-β1 levels in the culture supernatants of the calvarial cells from the homozygotes were significantly higher than those from wild-type mice. Although the skull and femur are sclerotic in CED, these phenotypes were not observed in p.C225S homozygous mice. These results suggest that human and mouse bone tissue react differently to TGF-β1. These findings are useful to pharmacological studies using mouse models in developing drugs that will target TGF-β signaling.
Key words: Camurati-Engelmann disease, ENU-mutagenized mice, TGF-β1

Introduction

Camurati-Engelmann disease (CED, OMIM 131300) is a rare bone disorder in humans with autosomal dominant inheritance [12]. Radiologically, it is characterized by hyperostosis and sclerosis of long bone diaphyses and the skull base. Symptoms include bone pain, muscle weakness, a waddling gait, and fatigue. Mutations in the gene (*TGFB1*) encoding transforming growth factor-β1 (TGF-β1) are the causative factor for CED [10, 13].

TGF-β1 is a multifunctional cytokine that controls cell growth, differentiation, and morphogenesis in many different types of cell [14, 16]. In skeletal tissue, TGF-β1 couples bone formation with resorption by regulating the function of osteoblasts and osteoclasts [5, 6, 23]. Mature TGF-β1 is a 25 kDa homodimer derived by intracellular proteolytic processing of the propeptide [1, 8, 28]. Latency-associated peptide (LAP) is the cleaved N-terminal propeptide and remains non-covalently bound as a dimer to the mature TGF-β1 dimer, forming

the small latent complex (SLC). SLC inhibits TGF-β1 signaling by preventing the binding of mature TGF-β1 to its receptor. TGF-β1 and its receptors are ubiquitously expressed, and latency is an important mechanism for controlling its biological effects. Most CED-causing mutations are concentrated in exon 4 of *TGFB1* and occur at or close to the cysteine residues at positions 223 and 225 (Cys^{223} and Cys^{225}), which are involved in the formation of disulfide bounds between the LAP dimmer subunits [10, 13]. Studies using CED fibroblasts showed that the mutant cells produce more active TGF-β1 than the wild-type cells [11, 20]. CED-causing mutations are considered to destabilize the disulfide bridging between LAP dimmer subunits, destabilizing SLC and prematurely activating TGF-β1. However, the relationship between the altered TGF-β1 signaling and CED pathogenesis has not been fully elucidated.

A transgenic mouse model that exhibits CED-like bone defects has been established [23]. This model was generated by overexpressing TGF-β1 mutant proteins carrying a CED-causing mutation (p.H222D) in osteoblasts under the control of a 2.3-kb type I collagen promoter. However, a mutant mouse with an endogenous *Tgfb1* mutation and CED-like skeletal phenotypes has not been reported yet. In this study, we performed a *Tgfb1* mutation screening of an ENU-mutagenized mouse genomic DNA library and identified a p.C225S mutation in a position corresponding to the position of the CED-causing p.C225R mutation.

Materials and Methods

ENU mutagenesis and Tgfb1 mutation screening

Mouse ENU mutagenesis was performed as described at http://www.brc.riken.go.jp/lab/gsc/mouse/ and in previous reports [7, 15]. Briefly, ENU (total dosage of 150–250 mg/kg) was administered to male C57BL/6J mice, and these males were crossed with DBA/2J females to produce first generation (G1) offspring. A genomic DNA library for all G1 males was constructed. High-throughput mutation screening of the ENU-mutagenized genomic DNA library was performed as previously described [9, 21]. Because CED mutations are concentrated in exon 4 of *TGFB1*, we searched exon 4 and its flanking region for mutations.

Mouse recovery from frozen sperm

A mutant mouse line possessing a p.C225S mutation

($Tgfb1^{C225S}$) was recovered from G1 frozen sperm using conventional *in vitro* fertilization and embryo transfer methods. The congenic $Tgfb1^{C225S}$ strain was backcrossed onto a C57BL/6J background over seven generations. Experimental protocols were approved by the Animal Experiments Committees of RIKEN Tsukuba Institute and Iwate University. $Tgfb1^{C225S}$ genotyping was performed by direct sequencing or restriction fragment length polymorphism (RFLP) analysis of the PCR products amplified using the following primers: 5′-TCTTCCCCCTCATATCCTCA-3′ and 5′-ACAGCCTCGGAGAGAGACAG-3′. The PCR products were electrophoresed after *Xsp*I restriction digestion.

Construction of expression vectors

Total RNA was extracted from mouse embryos at embryonic day 18.5 using ISOGEN (Nippon Gene, Tokyo, Japan) and was reverse transcribed using a Multi-Scribe Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, USA). The entire coding region of *Tgfb1* cDNA was amplified via PCR and subcloned into the *Eco*RI/*Not*I sites of the pcDNA3.1 (+) vector. Mutant *Tgfb1* cDNAs with c.C225S or c.C225R were generated by PCR-based mutagenesis. The inserted fragments and the introduced mutations were confirmed by sequencing. PCR conditions and primer sequences used for cDNA cloning and PCR-based mutagenesis are available upon request.

Reporter gene assays

HEK293 cells were grown to 70%–80% confluence in 24-well multiplates and were transfected with plasmid DNA mixtures using FUGENE6 Transfection Reagent (Roche Diagnostics, Rotkreuz, Switzerland). The DNA mixture contained SBE4-luc (100 ng) [18], pcDNA3.1 (+) expression vectors (200 ng), and a reference vector, pRL-TK (1 ng). Forty-eight hours after transfection, cells were harvested, and luciferase activities were measured using the PG-DUAL-SP reporter assay system (Toyo Ink, Tokyo, Japan) and a Lumat LB 9507 luminometer (Berthold, Bad Wildbad, Germany). Relative luciferase activity was calculated after normalizing to Renilla luciferase activity, which was expressed by the reference vector.

Western blot analysis

COS-7 cells were grown to 70%–80% confluence in

6-well multiplates and were transfected with 1 μg of pcDNA3.1 (+) expression vectors. Cells were maintained in Opti-MEM I for 48 h after transfection. The collected supernatants were concentrated 10-fold using a Vivaspin 500, 10 K MWCO, centrifugal concentrator (Vivascience AG, Hannover, Germany). Proteins were separated using SDS-PAGE gels and transferred to PVDF membranes. After blocking with 5% BSA in TBS-T, membranes were incubated with a TGF-β primary antibody (#3711, Cell Signaling Technology, Danvers, MA, USA) and then with goat anti-rabbit polyclonal secondary antibody IgG conjugated with horseradish peroxidase (Upstate Biotechnology, Lake Placid, NY, USA). Bands were visualized by chemiluminescent detection using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA).

X-ray and pQCT analyses

Femurs and skulls were dissected from sacrificed mice and fixed with 70% ethanol. Radiographs were obtained using a TRS-1005 soft X-ray apparatus (Sofron, Tokyo, Japan). Femoral cortical bone density and cortical thickness were measured by peripheral quantitative computed tomography (pQCT) analysis using an XCT Research SA computed tomography Scanner (Stratec Medizintechnik, Pforzheim, Germany).

Primary culture of calvarial osteoblasts and measurement of TGF-β1 levels

Calvariae were dissected from mice at postnatal day 7. Calvarial osteoblasts were isolated during four sequential 15-min digestions in an enzyme mixture containing 0.05% trypsin (Gibco BRL, Gaithersburg, MD, USA) and 0.2% dispase II (Godo Shusei, Tokyo, Japan) at 37°C. Fractions 2–4 were collected and plated in 6-well multiplates. When confluent, the cells were maintained in serum-free Opti-MEM I for 72 h. The collected supernatants were concentrated 20-fold using a Vivaspin 500, 10K MWCO, centrifugal concentrator. The amount of mature and total TGF-β1 in the supernatants was determined using a Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA). Total TGF-β1 was measured after the activation of latent TGF-β1. Activation was achieved by acidification of supernatants with 1 N HCl for 10 min and neutralization with 1.2N NaOH/0.5 M HEPES.

Statistical analysis

Statistical analyses were performed using Mac Statis-

tical Analysis ver. 2.0 (Esumi, Tokyo, Japan). Differences among multiple groups were compared by one-way ANOVA followed by the Tukey-Kramer test for multiple comparisons. Student's *t*-test was used to analyze differences between two groups. *P*-value<0.05 was judged as significant.

Results

Identification of a novel Tgfb1 missense mutation (p. C225S) in ENU-mutagenized mice

High-throughput mutation screening of *Tgfb1* exon 4 in an ENU-mutagenized mouse genomic DNA library revealed three mutations in approximately 7,400 G1 genomic DNA samples. The three mutations included one missense and two intronic mutations. The missense mutation (c.673T>A) was predicted to substite cysteine residue with serine residue at position 225 (p.C225S) (Fig. 1A) and was confirmed by PCR-RFLP analysis (Fig. 1B) and cDNA sequencing (data not shown). The human p.C225R mutation was identified in CED families; interestingly, the two mutations substitute the same Cys[225] residue of TGF-β1. We named this novel *Tgfb1* allele *Tgfb1*[C225S]. The identified intronic mutations were not predicted to alter gene function, because they were located far from exon–intron boundaries.

Functional analyses of p.C225S mutant proteins

To examine the effect of the p.C225S and p.C225R mutations in mouse *Tgfb1* on TGF-β activity, we constructed wild-type and mutant expression vectors. To examine extracellular secretion, we measured TGF-β1 protein concentrations in cell lysates and culture supernatants from COS-7 cells transfected with wild-type, p.C225S mutant, and p.C225R mutant expression vectors using western blot analysis. Single bands corresponding to the precursor and mature TGF-β1 were detected in the lysates and culture supernatants of cells transfected with the three expression vectors (Fig. 2A). There were no differences in the level of protein expression, demonstrating that p.C225S and p.C225R mutant proteins were correctly secreted by COS-7 cells.

Next, we examined the effects of the mutations on the activation of intracellular TGF-β signaling by reporter gene analysis. Because Smad2/3 are critical intermediates in TGF-β signaling [16], we used SBE4-luc, a Smad2/3-dependent reporter gene [18]. All TGF-β1 constructs enhanced reporter gene expression, but the
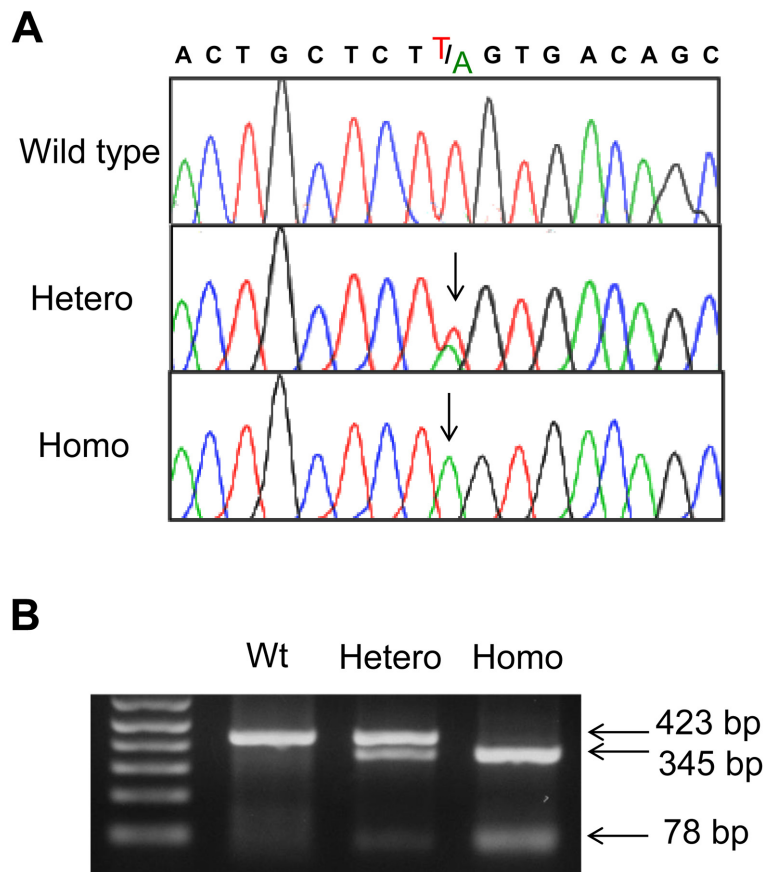
A

A C T G C T C T T/A G T G A C A G C

Wild type

Hetero

Homo

B

Wt   Hetero   Homo

← 423 bp
← 345 bp

← 78 bp

Fig. 1. Identification of a *Tgfb1* missense mutation (p.C225S) in ENU-mutagenized mice. (A) Direct sequence chromatograms of PCR products amplifying the regions surrounding exon 4 of *Tgfb1*. The mutation (c.673T>A) is indicated by the arrow and predicted to substitute cysteine with serine at position 225 (p.C225S). (B) Electrophoresis image of RFLP analysis identifying the c.673T>A missense mutation. The mutation generates an *Xsp*I recognition site (CTAG). *Xsp*I digestion of c.673T>A-containing 423 bp PCR products produces 345 bp and 78 bp bands.

strongest expression was induced by the two mutants (Fig 2B). There was no difference in the ability of the two mutants to enhance reporter gene expression. These results indicate that the Cys^{225} residue of mouse TGF-β1 is involved in the activation of latent TGF-β1 and that TGF-β signaling is equally enhanced by the p.C225S and p.C225R mutations.

Bone phenotypes of Tgfb1^{C225S} mice

   Functional analyses of the mutant proteins indicated that *Tgfb1*^{C225S} mice are identical to mice carrying the p.C225R mutation that corresponds to the known CED-causing mutation. *Tgfb1*^{C225S} mice were recovered from G1 frozen sperm and confirmed to have no other mutations except p.C225S in the coding region of *Tgfb1*. The

hallmark of CED is the cortical thickening of long bone diaphyses. In some cases, sclerotic changes are observed in the skull base. The onset of CED usually occurs during childhood and almost always before the age of 30 [12]. Therefore, we examined the bone phenotypes of *Tgfb1*$^{C225S/C225S}$ mice (p.C225S homozygotes) after adolescence. X-ray analysis of the skull and femur at 6 months of age showed no radiolucent differences between wild-type and *Tgfb1*$^{C225S/C225S}$ mice (Fig. 3A). The same result was obtained at 14 months of age (data not shown). Furthermore, we measured the femoral cortical bone density and cortical thickness at 6 months of age by pQCT analysis (Fig. 3B). *Tgfb1*$^{C225S/C225S}$ mice had cortical bone qualities similar to those of wild-type mice. Abnormally long limbs in proportion to height as well
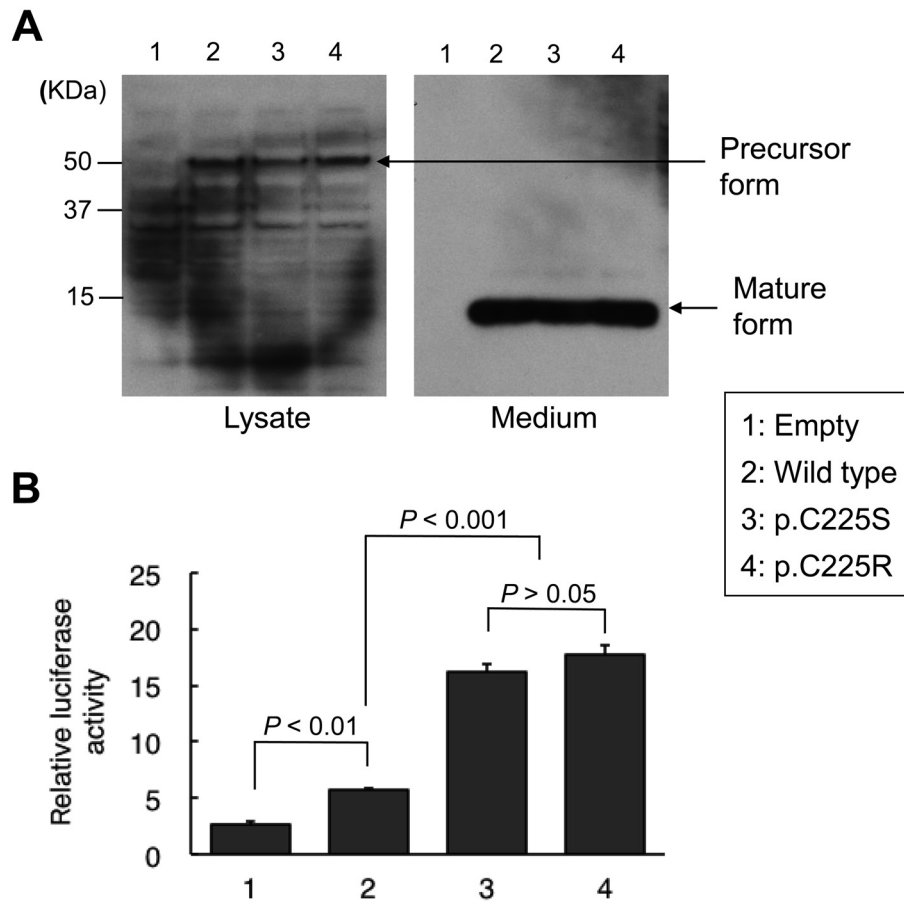
Fig. 2. Molecular characterization of the p.C225S mutant protein. (A) Western blot analysis using lysates and culture supernatants from COS-7 cells transfected with expression vectors for empty, wild-type TGF-β1, p.C225S mutant, and p.C225R mutant under reducing conditions. Bands corresponding to precursor and mature TGF-β1 were detected in the lysates and supernatants of cells transfected with three expression vectors. (B) Reporter gene assay to measure TGF-β signaling. The Smad2/3-dependent reporter vector, SBE-luc, was co-transfected with the respective expression vectors. Higher expressions of the reporter gene were induced by p.C225S and p.C225R mutants than by the wild-type protein. There was no difference in the level of reporter gene expression induced by the two mutants. Value represent means ± SD (n=5). P-values were determined by one-way ANOVA followed by the Tukey-Kramer test for multiple comparisons. Similar results were obtained in three independent experiments, and representative data are shown.

as reduced muscle mass and body fat have been reported in CED patients, but the growth and physique of $Tgfb1^{C225S/C225S}$ mice were normal at 6 and 14 months of age (data not shown). No gross abnormalities were observed in the mutant mice.

Finally, mature and total TGF-β1 levels were measured in the culture supernatants of calvarial osteoblasts prepared from mice at postnatal day 7 (Fig. 4). Mature TGF-β1 levels were higher in $Tgfb1^{C225S/C225S}$ cells than in wild-type cells, whereas total TGF-β1 levels were not significantly different between the two cell types.

Discussion

Through high-throughput mutation screening in an ENU-mutagenized mouse genomic DNA library, we identified a novel $Tgfb1$ mutation (p.C225S) in mice that corresponds to the known CED-causing mutation (p. C225R) found in humans. To explore the effects of this mutation, we generated a $Tgfb1^{C225S}$ mutant mouse. Although mature TGF-β1 secretion by $Tgfb1^{C225S/C225S}$ calvarial osteoblasts was higher, no CED-like bone defects were observed. In contrast, one copy of the *TGFB1*
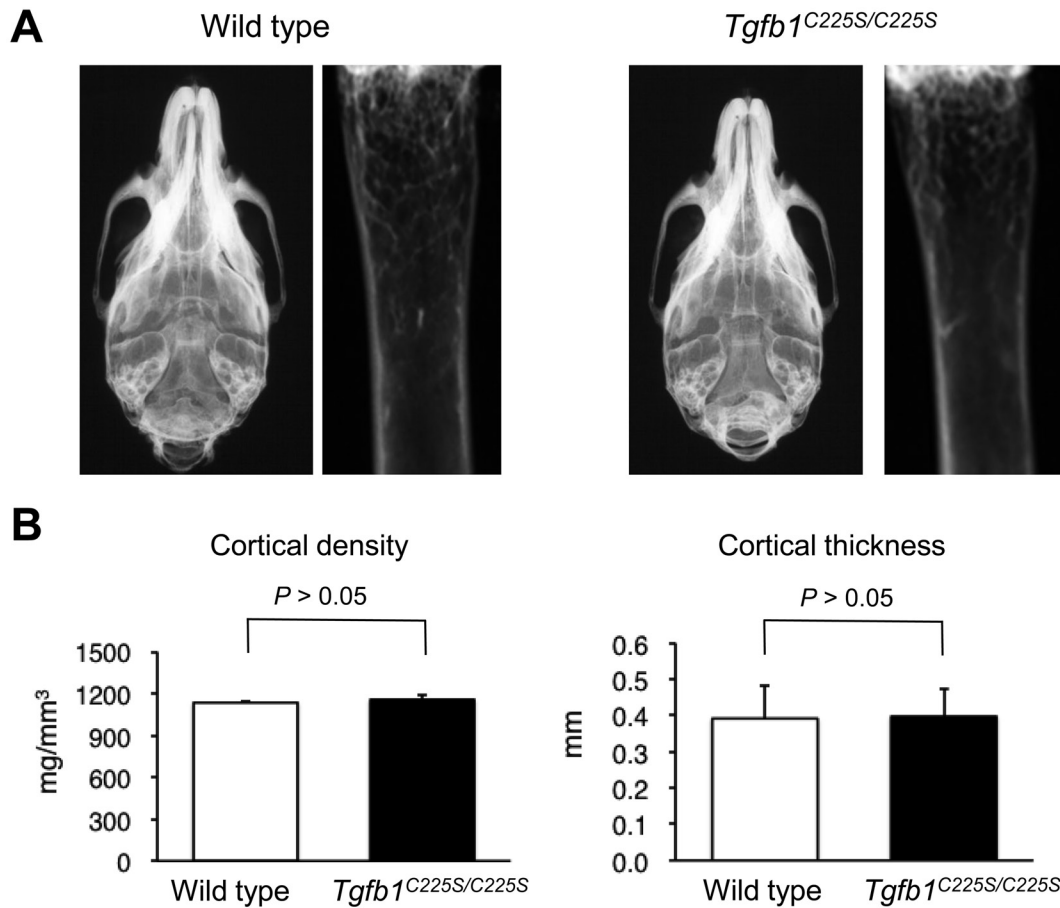
A

Wild type   *Tgfb1*^C225S/C225S^

B

Cortical density   Cortical thickness

Fig. 3. Bone phenotypes of *Tgfb1*^C225S/C225S^ mice. (A) X-ray images of the skull and femur at 6 months of age. (B) Measurement of femoral cortical bone density and cortical thickness at 6 months of age. Values are means ± SD (n=4). *P*-values were determined by Student's *t*-test. *Tgfb1*^C225S/C225S^ mice had a normal bone phenotype.

Mature TGF-β1   Total TGF-β1
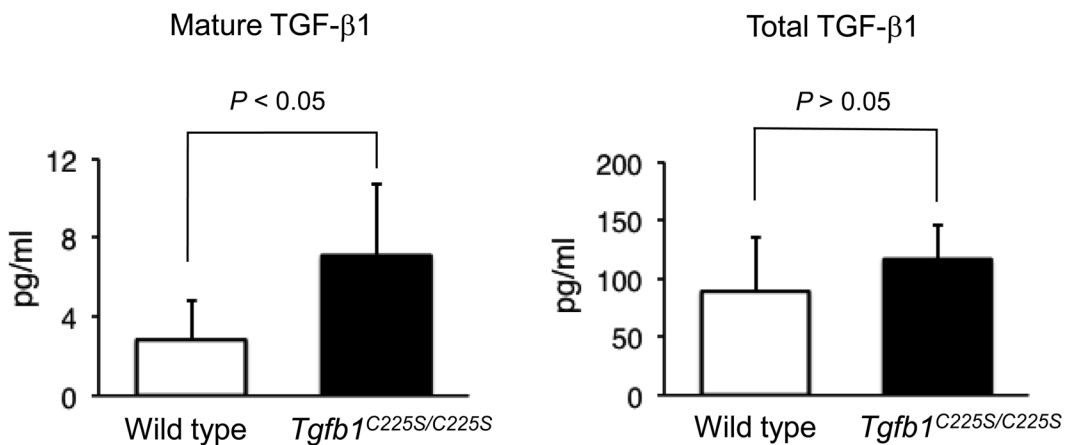

Fig. 4. Mature and total TGF-β1 levels in the culture supernatants of calvarial osteoblasts prepared from mice at postnatal day 7. Values represent means ± SD (n=6). *P*-values were determined by Student's *t*-test. Mature TGF-β1 levels were significantly higher in *Tgfb1*^C225S/C225S^ cells than those in wild-type cells. Similar results were obtained in two independent experiments, and representative data are shown.

mutation is sufficient to cause bone defects in humans; CED is inherited as a dominant trait [10, 13]. These findings suggest that human and mouse bone tissues respond differently to TGF-β1. The CED-causing p.H222D mutation causes severe CED-like bone defects when overexpressed in the osteoblast lineage of mice under the control of the type I collagen promoter [23]. Transgenic production in this mouse model is high due to the strong activity of the type I collagen promoter. Based on these observations, we predict that the expression level of active TGF-β1 in $Tgfb1^{C225S/C225S}$ mice is below the threshold to cause bone defects in mice.

In most cells, LAP is linked to the latent TGF-β binding protein (LTBP), forming the large latent complex (LLC) [1, 19]. LTBP is required for efficient secretion and correct folding of mature TGF-β. The LAP subunit of TGF-β1 has three cysteine residues (Cys^{33}, Cys^{223}, and Cys^{225}), and the Cys^{33} residue of LAP and a cysteine residue in LTBP are linked by a disulfide bonds. Cys^{33} was substituted by serine in $Tgfb1$ mutant mice ($Tgfb1^{C33S/C33S}$), and this reduced the levels of mature TGF-β1, as well as inflammatory and tumorigenic phenotypes reported in $Tgfb1^{-/-}$ mice [22, 27]. These findings suggest that covalent binding between LAP and LTBP is required for the activation of TGF-β1. Integrin $\alpha_v\beta_6$ is also reported to be an activator of latent TGF-β1 and TGF-β3 [2, 17, 26]. LAP contains an Arg-Gly-Asp (RGD) sequence that binds to integrin $\alpha_v\beta_6$. Mutation of this RGD sequence to Arg-Gly-Glu (RGE) in $Tgfb1^{RGE/RGE}$ mice recapitulated the phenotypes of $Tgfb1^{-/-}$ mice, suggesting that this RGD-binding integrin activates latent TGF-β1 [26]. LTBP-1 is required for this integrin-mediated activation, although integrin $\alpha_v\beta_6$ recognizes the RGD sequence of LAP [2]. The phenotypes of three $Tgfb1$ mutant mice ($Tgfb1^{C225S/C225S}$, $Tgfb1^{C33S/C33S}$, and $Tgfb1^{RGE/RGE}$) indicate that the major latent TGF-β1–activating mechanisms involve LTBP and that those mediated by SLC alone are negligible in mice.

During bone remodeling, TGF-β1 is released from the bone matrix and activated by osteoclast-mediated bone resorption, thereby creating a gradient of mature TGF-β1. This gradient induces the migration of mesenchymal stem cells (MSCs) to the recently resorbed bone surfaces for osteoblast differentiation and new bone formation [5, 23]. In transgenic mice, overexpression of the CED-causing p.H222D mutation in osteoblasts disrupted the targeted recruitment of MSCs to sites of bone remodeling because the TGF-β1 gradient was lost [23].

Furthermore, it has been reported that TGF-β signaling promotes commitment to the osteoblast lineage, osteoprogenitor proliferation, and early-stage osteoblast differentiation, while also inhibiting late-stage osteoblast differentiation and mineralization [4]. Osteoclast-specific TGF-β receptor 2 knockout mice have osteopenia due to reduced osteoblast numbers, although osteoclast numbers and activity are not affected [25]. The reduced level of TGF-β-induced Wnt1 in osteoclasts may contribute to the observed phenotype. These findings suggest that osteoclast responses to TGF-β are also involved in coupling bone resorption to bone formation.

Enhanced TGF-β signaling has been reported in many other connective tissue diseases, including Marfan syndrome, Loeys-Dietz syndrome, and Shprintzen-Goldberg syndrome [3, 24]. Attenuating TGF-β signaling may be an effective therapeutic strategy for these diseases. The findings of this study provide useful information for pharmacological studies using mouse models to develop drugs that target TGF-β signaling.

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