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Establishment of a novel model of chondrogenesis using murine embryonic stem cells carrying fibrodysplasia ossificans progressiva-associated mutant ALK2

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ABSTRACT

Fibrodysplasia ossificans progressiva (FOP) is a genetic disorder characterized by heterotopic endochondral ossification in soft tissue. A mutation in the bone morphogenetic protein (BMP) receptor ALK2, R206H, has been identified in patients with typical FOP. In the present study, we established murine embryonic stem (ES) cells that express wild-type human ALK2 or typical mutant human ALK2 [ALK2(R206H)] under the control of the Tet-Off system. Although wild-type ALK2 and mutant ALK2(R206H) were expressed in response to a withdrawal of doxycycline (Dox), BMP signaling was activated only in the mutant ALK2(R206H)-expressing cells without the addition of exogenous BMPs. The Dox-dependent induction of BMP signaling was blocked by a specific kinase inhibitor of the BMP receptor. The mutant ALK2(R206H)-carrying cells showed Dox-regulated chondrogenesis *in vitro*, which occurred in co-operation with transforming growth factor-β1 (TGF-β1). Overall, our ES cells are useful for studying the molecular mechanisms of heterotopic ossification in FOP *in vitro* and for developing novel inhibitors of chondrogenesis induced by mutant ALK2(R206H) associated with FOP.

1. Introduction

Fibrodysplasia ossificans progressiva (FOP; OMIM135100) is a rare hereditary disorder characterized by postnatal, progressive heterotopic endochondral ossification in the skeletal muscle [1–3]. The *ACVR1* gene, on chromosome 2q23–24, encodes ALK2, which is one of the receptors for bone morphogenetic proteins (BMPs) and has been identified as the gene responsible for FOP [4]. In familial and sporadic cases, typical FOP patients have a recurrent substitution mutation from arginine to histidine at codon 206 (p.R206H) in ALK2 [4]. More than 10 additional mutations in ALK2 have been found in patients with FOP variants [5–7]. The over-expression of ALK2(R206H) in cultured cells *in vitro* induces events involved in BMP signaling, such as the phosphorylation of Smad1 and Smad5 and activation of BMP target-gene transcription without ligand stimulation [8–11]. These findings suggest that FOP is the first known case of a natural gain-of-function mutation in a BMP receptor.

Skeletal tissues are formed through two different but related processes: intramembranous ossification and endochondral ossification [2,12]. In intramembranous ossification, undifferentiated mesenchymal cells differentiate into osteoblasts, which form bone tissue. By contrast, in endochondral ossification, the cells differentiate into chondrocytes to form the cartilaginous templates of future bone tissue. The mature cartilage in the templates induces osteoblast differentiation in the perichondrium and is subsequently replaced by bone tissue with vascular invasion [13]. In FOP, heterotopic ossification is formed via the endochondral process [14]. However, the cellular process of heterotopic ossification in FOP is still unclear because invasive examinations and treatments are prohibited in FOP patients to prevent acute heterotopic

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ossification. Recently, knock-in mice of mutant ALK2(R206H) have been established; however, the mice died after birth [15]. In the present study, we established mouse embryonic stem (ES) cell lines expressing wild-type human ALK2 or mutant ALK2(R206H) under the control of the Tet-Off system. The ES cells that carry the mutant ALK2(R206H) were able to activate BMP signaling in response to the withdrawal of doxycycline (Dox) from the cultures without the addition of exogenous BMP ligands, whereas ES cells carrying the wild-type ALK2 did not show the same effect. Moreover, Dox-regulated chondrogenesis was observed in the ES cells carrying ALK2(R206H) in the absence of Dox. Our ES cells will be useful for studying the molecular mechanisms of heterotopic ossification in FOP *in vitro* and developing novel inhibitors of chondrogenesis induced by the ALK2(R206H) mutant associated with FOP.

2. Materials and methods

2.1. Cell culture

The mouse ES cell line EBRTcH3 was maintained in Glasgow minimal essential medium (GMEM) (Sigma–Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Nichirei, Tokyo, Japan) in the presence of leukemia inhibitory factor (LIF, Nacalai Tesque, Kyoto Japan) [16]. This cell line was modified at the *ROSA26* locus to express an exogenous gene under the control of the Tet-Off system [16,17]. Two types of targeting vectors carrying human ALK2(WT) and ALK2(R206H) cDNA were constructed in the exchange vector pZhCSfi [16]. One of the vectors was co-transfected in EBRTcH3 cells with a Cre-DNA recombinase expression vector, and the cells were selected in culture media containing zeocin in the presence of Dox [16]. The hygromycin-sensitive phenotype was confirmed for the expected recombination at the *ROSA26* locus in the subclonal cells.

To induce chondrogenesis, the ES cells were inoculated at 1×10^3 cells/well in a 96-well PrimeSurface plate (Sumitomo Bakelite, Tokyo, Japan) in the presence of Dox and maintained for 5 days to form embryoid bodies (EBs). The EBs were dissociated by Accutase (GIBCO®, Life Technologies, Grand Island, NY, USA) and inoculated at 3×10^5 cells/tube in 15 ml Stemfull centrifuge tubes (Sumitomo Bakelite) with 0.5 ml of chondrogenic medium (Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 containing 0.1 μM dexamethasone, 50 μM L-ascorbic acid 2-phosphate, 40 μg/ml L-proline, 1 mM sodium pyruvate and 1% ITS) in the presence and absence of 10 ng/ml of TGF-β1 (PeproTech, Rocky Hill, NJ, USA) and/or 50 ng/ml of BMP-4 (R&D Systems, Minneapolis, MN). The cells were cultured as pellets for 2 or 3 weeks to induce chondrogenesis as previously described [18]. The culture medium was replaced with fresh medium once every 2–3 days.

2.2. Analysis of human ALK2 expression by immunohistochemical staining and flow cytometry

The expression of the V5-epitope-tagged human ALK2 was examined by immunohistochemical analysis as previously described [19]. The ES cells were stained with a mouse monoclonal antibody against the V5-epitope tag (clone V5005, Nacalai Tesuque) and Alexa 594-labeled goat polyclonal secondary antibody against mouse immunoglobulin G (IgG; Molecular Probes®, Life Technologies). A BZ-9000 fluorescence microscope (Keyence, Tokyo, Japan) was used for fluorescence analysis.

A flow cytometry analysis was performed using a goat polyclonal antibody against the extracellular domain of ALK2 (R&D Systems), which was labeled by Alexa 647 using a kit (Molecular Probes®, Life Technologies). The stained cells were analyzed by FACSCalibur™ (BD Biosciences, San Jose, CA, USA).

2.3. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The expression levels of specific target mRNAs were determined by real-time RT-PCR [20]. The total RNA was prepared using a NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) and reverse-transcribed with Superscript III (Invitrogen, Life Technologies) according to the manufacturer's instructions. Quantitative RT-PCR (qRT-PCR) was performed using SYBR Premix Ex Taq (TaKaRa, Shiga, Japan) with a Thermal Cycler Dice TP800 (TaKaRa). The primers that were used are listed in Supplemental Table 1. The data are expressed as the expression levels relative to that of Atp5f1.

2.4. Luciferase reporter assay

BMP signaling was determined in a luciferase reporter assay using the BMP-specific reporter Id1WT4F-luc, which is driven by tandem copies of a Smad-binding element in the *Id1* gene [10,11,21]. The ES cells inoculated in a 96-well plate were transfected with 40 ng of Id1WT4F-luc, 10 ng of phRL-SV40 and 150 ng of empty plasmid DNA using 0.5 μl of Lipofectamine 2000 reagent (Invitrogen, Life Technologies) following the manufacturer's instructions, and the culture medium was changed after 2.5 h. The luciferase assay was performed on day 1 using the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA), and the data were expressed as the luciferase activity of firefly relative to that of *Renilla*.

2.5. Western blot analysis

Whole-cell extracts were prepared using lysis buffer containing a 1× protease inhibitor cocktail (Roche, Indianapolis, IN, USA) [8,11,19]. The extracts were then separated via sodium dodecyl sulfate-containing polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. The proteins were detected by immunoblotting with antibodies against the following proteins: phosphorylated Smad1/5 (rabbit monoclonal antibody, Cell Signaling Technology, Beverly, MA, USA), the V5-tag (mouse monoclonal antibody, clone V5005, Nacalai Tesuque) and tubulin (rabbit polyclonal antibody, Cell Signaling Technology). The target proteins were detected using a horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Chemiluminescence was detected using the ChemiDoc XRS+ System (Bio-Rad, Hercules, CA, USA).

2.6. Statistical analysis

The results are expressed as the mean ± SD ($n = 3$), and comparisons were performed using an unpaired Student's *t*-test. Statistical significance was indicated as * ($p < 0.05$) or ** ($p < 0.01$).

3. Results

3.1. Establishment of murine ES cells expressing human ALK2(WT) or ALK2(R206H) under the control of the Tet-Off system

To establish murine ES cells that express human ALK2 under the control of the Tet-Off system, we constructed exchange vectors carrying V5-tagged ALK2(WT) and ALK2(R206H) (Fig. 1A). Each vector was co-transfected with an expression vector carrying Cre-DNA recombinase in murine EBRTcH3 ES cells in which a Tet-Off system had previously been introduced at the *ROSA26* locus [16]. Among the Zeo^r and Hyg^s colonies, the expression of ALK2 in the presence

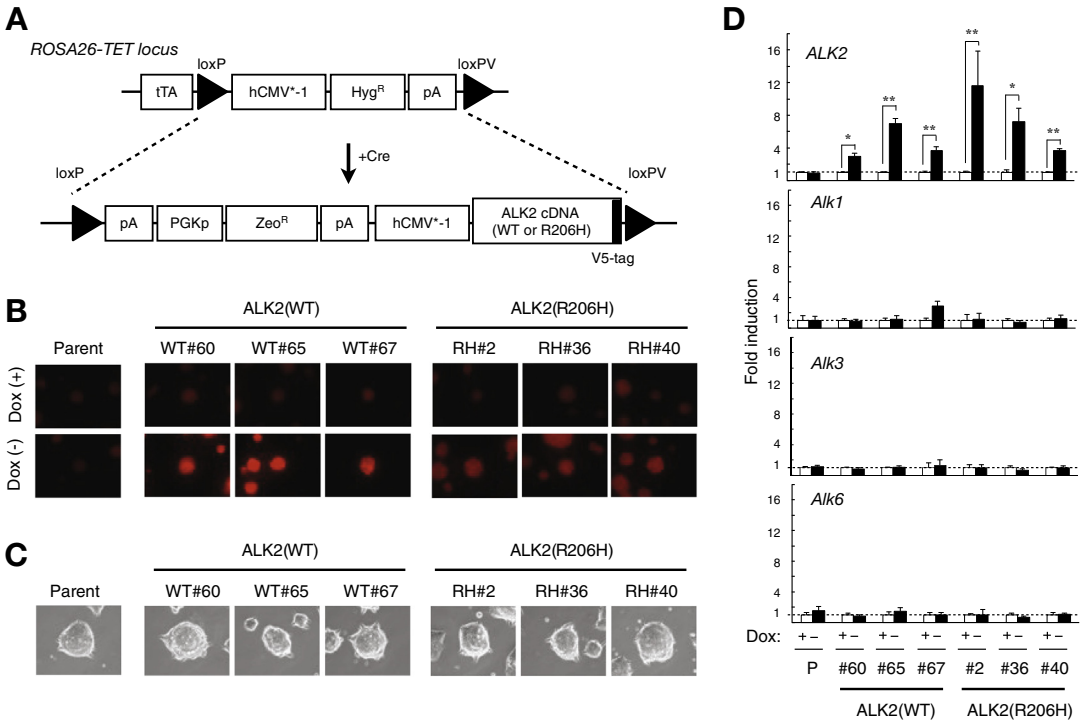

Fig. 1. Establishment of murine ES cell lines carrying mutant FOP-associated ALK2. (A) A schematic representation of the *ROSA26* locus and exchange vector for V5-tagged human ALK2(WT) and ALK2(R206H). (B) Immunohistochemical staining of ALK2(WT) and ALK2(R206H) using an antibody against the V5-tag. ES cells were cultured for 2 days in the presence [Dox(+)] and absence [Dox(−)] of 1 μg/ml Dox. The colonies that were formed were stained with an antibody against the V5-tag. (C) Colonies formed in parental ES cells and their subclones carrying ALK2(WT) or ALK2(R206H). Phase contrast views. (D) The specific induction of ALK2 mRNA in ES cells under the control of the Tet-Off system. The ES cells were cultured for 2 days in the presence (open bars) and absence (closed bars) of 1 μg/ml Dox. The expression levels of the mRNA of *ALK2*, *Alk1*, *Alk3*, *Alk6* were determined by qRT-PCR. Data were expressed in fold induction in the absence of Dox compared to the presence of Dox.

and absence of Dox was examined by immunohistochemical staining using an antibody against the V5-tag (Fig. 1B). Three independent clones (WT#60, WT#65 and WT#67; RH#2, RH#36 and RH#40) were chosen from the cultures transfected with the vectors carrying ALK2(WT) and ALK2(R206H), respectively (Fig. 1B). The colonies formed in the feeder-free cultures were indistinguishable from those of parental EBRTcH3 cells (Fig. 1C). The 6 subclones expressed human *ALK2* mRNA in the absence of Dox, whereas the parental ES cells did not (Fig. 1D, top panel). However, other BMP type I receptors, such as *Alk1*, *Alk3*, and *Alk6*, were not induced in those subclones, even in the absence of Dox (Fig. 1D).

3.2. BMP signaling is activated in the ES cells carrying ALK2(R206H) and not ALK2(WT) by the withdrawal of Dox

The flow cytometry analysis using an antibody against ALK2 confirmed that both wild-type and mutant ALK2 proteins were expressed on the cell surface in the absence of Dox (Fig. 2A), and there was no change in the fluorescence signals in the parental cells whether in the presence or absence of Dox (Fig. 2A). The expression of wild-type and mutant V5-tagged human ALK2 was detected with a Western blot analysis of the cells cultured in the absence of Dox (Fig. 2B, top panel). However, the phosphorylation of Smad1/5 was detected only in the mutant ALK2(R206H)-carrying subclones, such as RH#2, RH#36 and RH#40, which occurred in the absence of stimulation by exogenous BMPs (Fig. 2B middle panel). In these cells, a Smad-dependent BMP-specific luciferase reporter (Id1WT4F-luc) (Fig. 2C), and endogenous target genes of Smad1/5, such as *Id1* and *Id2* (Fig. 2D and E), were induced in the absence of Dox (Fig. 2D and E). The phosphorylation of Smad1/5 and induction of BMP-specific luciferase reporter activity in the mutant ALK2-carrying ES cells were completely suppressed by adding LDN-193189, which is a specific chemical inhibitor of BMP type I receptor kinases [22] (Fig. 3A and B). These results suggest that Dox-regulated BMP-specific signaling is activated by the expression of mutant ALK2(R206H) in the absence of Dox.

3.3. Chondrogenic differentiation of RH#2 cells, a subclone carrying mutant ALK2(R206H)

Chondrogenesis in murine ES cells *in vitro* is induced by simultaneous stimulation with transforming growth factor-β (TGF-β) and BMP in pellet cultures [23]. Using this procedure, we examined the chondrogenic differentiation capacity of mutant ALK2(R206H)-carrying RH#2 cells. In the pellet cultures, the expression of marker genes related to chondrogenesis, such as type II collagen (*Col2a1*), aggrecan (*Acan*) and type 10 collagen (*Col10a1*), was induced by stimulation with TGF-β1 and BMP-4 in the presence of Dox (Fig. 4A–C). Those levels were further increased in the absence of Dox (Fig. 4A–C). The differentiation of macroscopic chondrocytes was confirmed by alcian blue staining of sections of the pellets (Fig. 4D). Chondrogenic differentiation was also induced in the cultures maintained with TGF-β1 alone in the absence of Dox, although the mRNA levels were lower than those in the cultures stimulated with TGF-β1 and BMP-4 (Fig. 4E–G). WT#60 cells carrying the wild-type ALK2 did not differentiate into chondrocytes in the pellet cultures stimulated with TGF-β1 alone, even in the absence of Dox (data not shown). Chondrocytes stained with alcian blue were detected in the pellets maintained with TGF-β1 without BMP-4 in the absence of Dox, whereas they were not detected in the presence of Dox (Fig. 4H).

4. Discussion

In the present study, we established murine ES cells expressing wild-type or mutant human ALK2(R206H) under the control of the

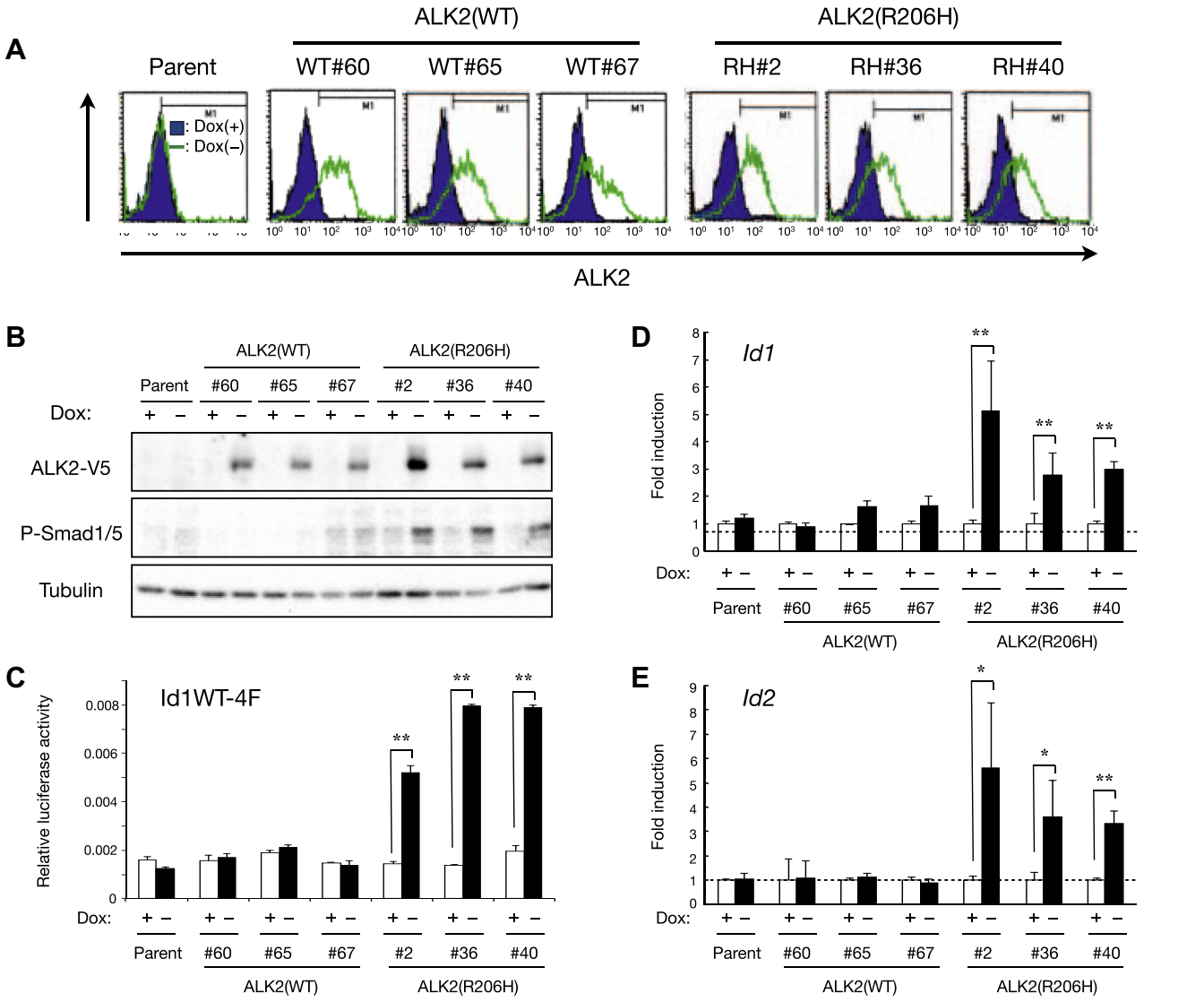

Fig. 2. Induction of ALK2(R206H) activates intracellular signal transduction of BMPs in ES cells. (A) Flow cytometry analysis of ALK2(WT) and ALK2(R206H) in ES cells. The ES cells that had been cultured for 2 days in the presence (blue areas) and absence (green lines) of 1 μg/ml Dox were stained with an antibody against ALK2 and analyzed by FACSCalibur™. (B) Western blot analysis of V5-tagged ALK2 and phosphorylated Smad1/5. ES cells were cultured for 2 days in the presence (+) and absence (−) of 1 μg/ml Dox. Whole-cell lysates were analyzed by Western blotting using antibodies against the V5-tag (ALK2-V5), phosphorylated Smad1/5 (P-Smad1/5) and tubulin. (C) Luciferase reporter assay in ES cells. The ES cells were transfected with a BMP-specific luciferase reporter, Id1WT4F-luc, and cultured overnight in the presence (open bars) and absence (closed bars) of 1 μg/ml Dox. (D and E) Expression of endogenous *Id1* and *Id2* mRNAs in ES cells carrying ALK2(R206H). The ES cells were cultured for 2 days in the presence (open bars) and absence (closed bars) of 1 μg/ml Dox. The expression levels of *Id1* and *Id2* mRNAs were determined by qRT-PCR. Data were expressed in fold induction in the absence of Dox compared to the presence of Dox. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Tet-Off system. ALK2(R206H) has been identified in patients with FOP and has been shown to be an activated BMP type I receptor [4,8–11]. Accordingly, the established subclones carrying mutant ALK2(R206H) were induced to activate intracellular signaling of BMPs in response to Dox withdrawal, whereas the subclones carrying ALK2(WT) were not induced. Moreover, RH#2 cells showed Dox-regulated chondrogenesis *in vitro* in co-operation with TGF-β1. The simultaneous stimulation of murine ES cells with TGF-β3 and BMP-4 has been shown to induce chondrogenesis *in vitro* [23]. In the cultures maintained with TGF-β1 alone, RH#2 cells differentiated into chondrocytes in the absence of Dox but not in the presence of Dox. These data suggest that the mutant ALK2(R206H) induced in the absence of Dox induced chondrogenesis of RH#2 cells in co-operation with TGF-β1.

Because biopsy or surgical treatments are prohibited in patients with FOP, this novel model of chondrogenesis may be useful for studying the molecular mechanisms of chondrogenesis induced by the mutant ALK2 *in vitro*. In addition, the Dox-regulated BMP signaling in the mutant ALK2(R206H)-expressing cells, including the RH#2 cells, was inhibited by adding LDN-193189, a specific small molecule chemical inhibitor of BMP type I receptor kinases. Other researchers, in addition to our group, have reported several small molecule inhibitors that can attenuate BMP signaling induced by mutant ALK2 in *in vitro* models [22,24–31]. Our ES cells carrying mutant human ALK2(R206H) may be useful for the screening of novel inhibitors of chondrogenesis induced by mutant ALK2, and these compounds could be applied to develop new treatments of FOP to prevent heterotopic ossification via the endochondral process.

Disclosure statement

The authors have nothing to disclose.

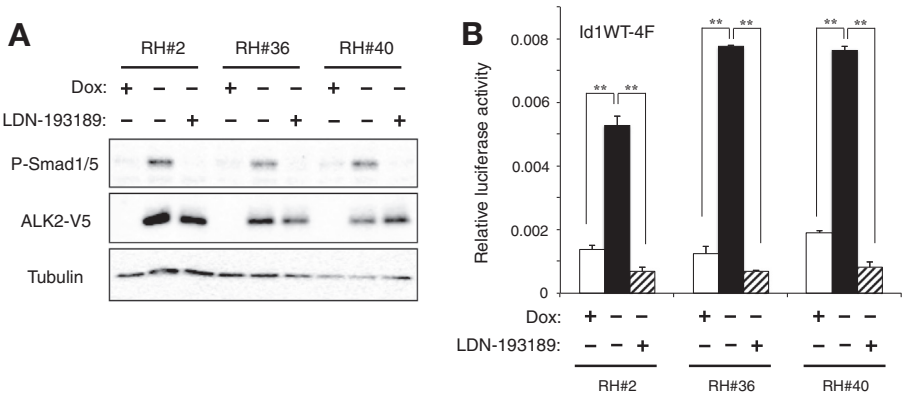

Fig. 3. LDN-193189, a specific small molecule chemical inhibitor of BMP type I receptors, inhibits Dox-dependent BMP signaling in ES cells carrying ALK2(R206H). (A) Western blot analysis of phosphorylated Smad1/5. ES cells carrying ALK2(R206H) were cultured for 2 days in the presence (+) and absence (−) of 0.1 μM LDN-193189 and 1 μg/ml Dox. Whole-cell lysates were analyzed by Western blotting using antibodies against phosphorylated Smad1/5 (P-Smad1/5), the V5-tag (ALK2-V5) and tubulin. (B) Luciferase reporter assay in ES cells carrying ALK2(R206H). The ES cells were transfected with a BMP-specific luciferase reporter, Id1WT4F-luc, and cultured overnight in the presence (+) and absence (−) of 0.1 μM LDN-193189 (hatched bars) and 1 μg/ml Dox (open bars).

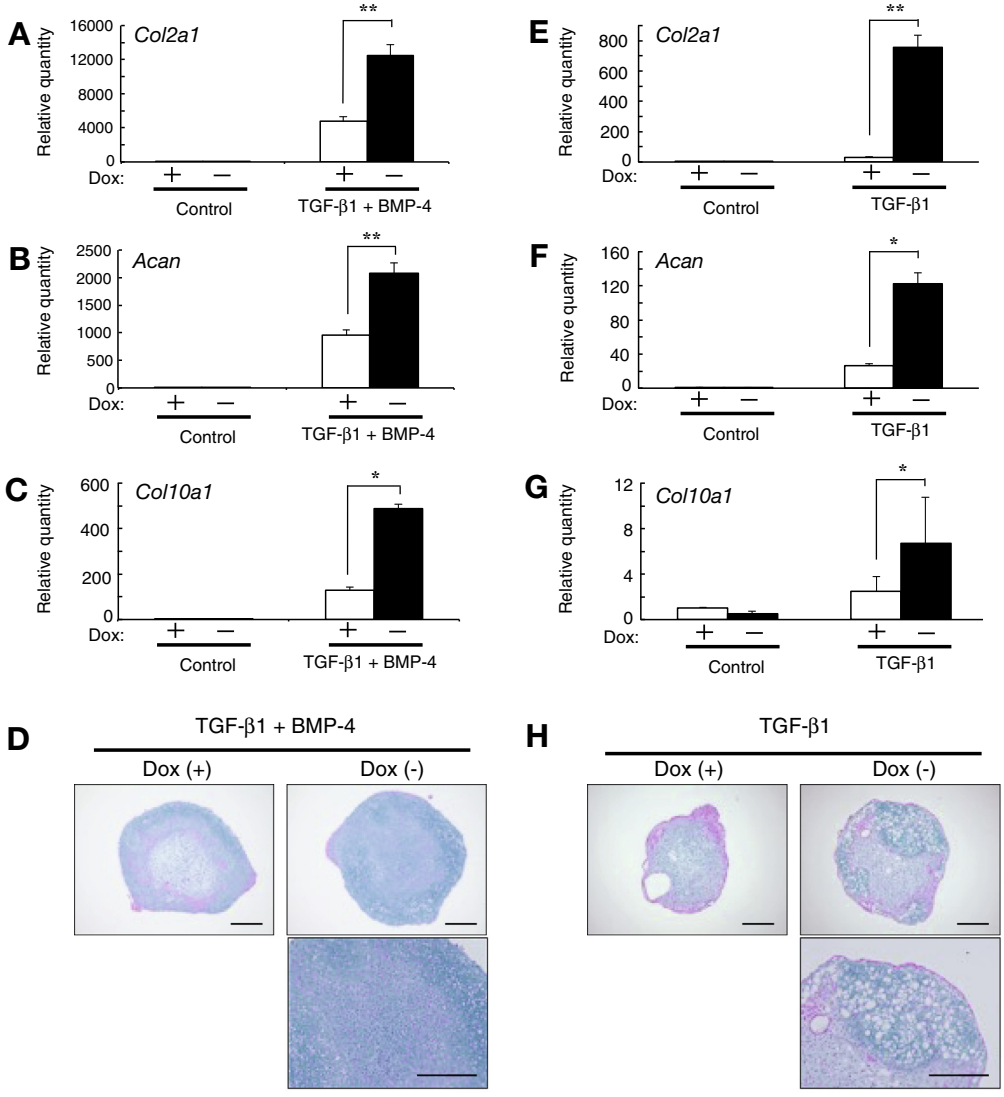

Fig. 4. Chondrogenic differentiation of RH#2 cells carrying ALK2(R206H). Chondrogenesis of RH#2 cells was induced as described in Section 2. The cells were maintained with 10 ng/ml of TGF-β1 and 50 ng/ml of BMP-4 (A–D) or 10 ng/ml of TGF-β1 alone (E–H) in the presence (open bars) or absence (closed bars) of 1 μg/ml Dox. The expression of *Col2a1* (A and E), *Acan* (B and F) and *Col10a1* (C and G) mRNA was determined by qRT-PCR. The relative mRNA levels were expressed in comparison to the control cultures in the presence of Dox. Macroscopic chondrogenesis of the pellets was evaluated by alcian blue staining of paraffin sections (D and H). Scale bars indicate 200 μm.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.012.

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