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Alk2 Regulates Early Chondrogenic Fate in Fibrodysplasia Ossificans Progressiva Heterotopic Endochondral Ossification

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Abstract

Bone morphogenetic protein (BMP) signaling is a critical regulator of cartilage differentiation and endochondral ossification. Gain-of-function mutations in ALK2, a type I BMP receptor, cause the debilitating disorder fibrodysplasia ossificans progressiva (FOP) and result in progressive heterotopic (extraskeletal) endochondral ossification within soft connective tissues. Here, we used murine mesenchymal progenitor cells to investigate the contribution of Alk2 during chondrogenic differentiation and heterotopic endochondral ossification (HEO). $Alk2^{R206H/+}$ (gain-of-function), $Alk2^{CKO}$ (loss-of-function), and wild-type mouse embryonic fibroblasts were evaluated for chondrogenic potential. Chondrogenic differentiation was accelerated in $Alk2^{R206H/+}$ cells, due in part to enhanced sensitivity to BMP ligand. In vivo, $Alk2^{R206H/+}$ cells initiated robust HEO and recruited wild-type cell contribution. Despite expression of other type I BMP receptors (Alk3 and Alk6), chondrogenesis of $Alk2^{CKO}$ cells was severely impaired by absence of Alk2 during early differentiation. Alk2 is therefore a direct regulator of cartilage formation and mediates chondrogenic commitment of progenitor cells. These data establish that at least one effect of

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ALK2 gain-of-function mutations in FOP patients is enhanced chondrogenic differentiation which supports formation of heterotopic endochondral bone. This establishes ALK2 as a plausible therapeutic target during early chondrogenic stages of lesion formation for preventing heterotopic bone formation in FOP and other conditions.

Keywords

Alk2; Chondrogenesis; Bone morphogenetic protein signaling; Fibrodysplasia ossificans progressiva; Endochondral ossification

Introduction

Normal development of the skeletal long bones occurs through endochondral ossification, the process by which a cartilage template is replaced by bone. Condensing mesenchymal progenitor cells proliferate and differentiate into chondrocytes, then are subsequently replaced by osteoblasts and bone [1]. Such endochondral bone formation also occurs in acquired and inherited forms of heterotopic endochondral ossification (HEO), the formation of bone within nonskeletal tissues [2, 3]. HEO formation follows the same general cellular processes that form skeletal bone; however, the specific cell types and mechanisms that modulate the cascade of ectopic ossification remain undetermined.

Fibrodysplasia ossificans progressiva (FOP; MIM #135100), an inherited disease of HEO, is an autosomal dominant disorder characterized by progressive endochondral bone formation within soft connective tissues [2, 4–6]. Patients develop highly inflammatory and vascular swellings (lesion flare-up) foreshadowing the apoptosis of affected skeletal muscle and connective tissue and repopulation by mesenchymal progenitor cells [7–9]. These progenitor cells differentiate to cartilage that transitions to mature mineralized bone tissue [10, 11]. All confirmed cases of FOP are caused by mutations in the *ACVR1* gene, which encodes ALK2, a type I bone morphogenetic protein (BMP) receptor [5, 6, 12]. Most FOP patients have the same specific R206H substitution in ALK2.

BMPs are extracellular ligands, part of the TGFβ superfamily, which exert their effects by binding to heteromeric complexes of type I and type II transmembrane serine/threonine kinase BMP receptors [13]. Signal transduction is mediated by four type I receptors (ALK2 [ACVR1], ALK3 [BMPR1A], ALK6 [BMPR1B], and ALK1 [ACVR1L]) and three type II receptors (ACTR2A, ACTR2B, and BMPR2). Upon ligand binding, the type II receptor phosphorylates the type I receptor GS domain. This facilitates activation of the neighboring protein kinase domain that subsequently induces downstream signal transduction by phosphorylating BMP-specific Smads (Smad1, Smad5, and Smad8) and/or components of the mitogen-activated protein kinase (MAPK) pathway to regulate gene transcription [14]. The $ALK2^{R206H}$ mutation in FOP appears to alter molecular interactions with the inhibitory protein FKBP12 and destabilize tertiary protein structure toward an activated conformation [15–18].

Signaling through BMPs and their receptors is a key regulator of chondrogenesis during development. BMP signaling is essential during mesenchymal cell condensation preceding

initial chondrocyte formation [19] and further participates in the proliferation and maturation of chondrocytes during the development of cartilage and bone [20, 21]. Canonical BMP signal transduction through Smad protein phosphorylation is indispensable for proper chondrogenesis [22].

The Alk2^{R206H} gain-of-function mutation enhances both canonical (phospho-Smad1/5/8) and noncanonical (phophop38) BMP signaling responses in the absence of ligand [17, 18, 23–25]. Furthermore, lesion biopsies from FOP patients and a R206H *Acvr1* knockin mouse model revealed that cartilage differentiation occurs within regions of fibroproliferation [2, 10, 11, 26]. The induction of chondrogenesis is therefore an important early step in the pathology of FOP. Effects of the Alk2^{R206H} mutation on in vitro chondrogenic differentiation were shown by over-expression of Alk2^{R206H} in chick limb bud micromass cultures [17]. These experiments supported chondrogenic regulation by Alk2; however, did not reproduce the heterozygous mutant state that occurs in patients and, since limb bud cells are committed toward chondrogenesis, could not evaluate the early critical commitment stages of progenitor cells.

In this study, we examined heterozygous Alk2^{R206H} expression in mesenchymal progenitor cells and determined that differentiation to cartilage in FOP patients is a direct consequence of heightened Alk2 signaling. We report that *Alk2*$^{R206H/+}$ mouse embryonic fibroblasts (MEFs) have enhanced sensitivity toward chondrogenesis both in vitro and in vivo. In addition, chondrogenesis by Alk2-deficient cells demonstrated that Alk2 is a key regulator of chondrogenic commitment.

MATERIALS AND METHODS

Animal Care and Use

Knockin *Alk2*$^{R206H/+}$ founder mice [26] generated germline *Alk2*$^{R206H/+}$ and *Alk2*$^{+/+}$ wild-type embryos for MEFs. Homozygous Alk2$^{floxed/floxed}$ mice [27] were bred to B6.Cg-Tg(CAG-cre/Esr1)5Amc/J mice [28] for Alk2$^{fl/fl}$;Cre/Esr1 (*Alk2*^{CKO}) embryos. C57BL/6-Tg(CAG-EGFP)10sb/J mice [29] were from Jackson Laboratory, Bar Harbor, ME, http://www.jax.org/. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at University of Pennsylvania.

Cell Culture

MEFs were isolated from 13.5 dpc mouse embryos [30]. With head and viscera removed, cells from each embryo were cultured individually in growth media (Dulbecco's modified Eagle's medium [DMEM, Gibco, Carlsbad, CA, http://www.lifetechnologies.com/] containing 10% fetal calf serum (FCS) [Invitrogen, Carlsbad, CA, http://www.lifetechnologies.com/]). MEFs at passages 3 and 4 were used for experiments. At least three individual embryo samples were used for experimental replicates.

For signaling assays, MEFs were cultured in DMEM without serum for 2 hours prior to adding 15 ng/ml hrBMP4 (R&D Systems, Minneapolis, MN, http://www.rndsystems.com/) for 1 hour. For analysis of prechondrogenic markers, MEFs were in growth media. For

growth curves, cells were plated at 1.5×10^4 cells per square centimeter and counted at time points by Trypan Blue (Gibco) exclusion.

Immunoblot Analysis

Total cell protein was recovered using M-PER containing Halt Protease and Halt Phosphatase Inhibitor Cocktails and quantified using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Wilmington, DE, http://www.fishersci.com/). Proteins were electro-phoresed through 10% SDS-polyacrylamide gels and transferred to nitrocellulose (Invitrogen). Membranes were blocked in 5% milk and incubated with primary antibodies against: phosphorylated Smad1/5/8 (1:750) and β-actin (1:3,000) (Cell Signaling Technology, Danvers, MA, http://www.cellsignal.com/), at 4°C overnight. Bound antibodies were detected with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:6,000) (Cell Signaling Technology) at room temperature, 1 hour. Detected proteins were imaged with Immobilon Chemiluminescent HRP Substrate (Millipore, Billerica, MA, http://www.millipore.com) and quantified using ImageJ Software.

RNA Isolation and Real-Time RT-PCR

RNA was isolated from undifferentiated MEFs in monolayer or MEFs in alginate spheres using TRIzol (Invitrogen) and quantified. cDNA was synthesized using High Capacity RNA-to-cDNA reagents (Applied Biosystems, Foster City, CA, http://www.lifetechnologies.com/). Real-time quantitative PCR reactions contained forward/reverse primers (0.37 μM, Supporting Information Table S1), cDNA (1:10 dilution), and Fast SYBR Green PCR Master Mix (Applied Biosystems); each sample was analyzed in triplicate. Target gene mRNAs were quantified from standard curves and normalized to the indicated housekeeping gene.

Cell Differentiation

For adipogenesis and osteogenesis, cells were seeded at 2.5×10^4 cells per square centimeter and cultured to confluence.

Adipogenic media (10% FCS, 1 μM dexamethasone, 10 mg/ml insulin, 0.5 mM IBMX [Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com/], and 10 μm rosaglitazone [Cayman Chemical, Ann Arbor, MI, www.caymanchem.com] in high glucose DMEM) were replenished every 3 days. For assays, cells were homogenized in TRIzol reagent, extracted for total protein, or formalin-fixed and stained with 0.2% oil red O (Sigma-Aldrich). Isopropanol extracted oil red O for quantification at 550 nm absorbance; samples were normalized to total protein of replicate wells.

Osteogenic media (10% FCS, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate (Sigma-Aldrich), and 100 ng/ml hrBMP4, in high glucose DMEM) were replenished every 3 days. For assays, cells were homogenized in TRIzol reagent, extracted for total protein, or stained with Alizarin red (Ricca Chemical, Arlington, TX, http://www.riccachemical.com/). Solution of 0.5 N HCl, 5% SDS extracted the deposited Alizarin red for quantification at 405 nm absorbance; samples were normalized to total protein of replicate wells.

For chondrogenesis, cell suspensions at 6.7×10^6 cells per milliliter in 1.2% alginate (Sigma-Aldrich) solution were extruded through 16-guage needles into 102 mM $CaCl_2$ (Thermo Fisher Scientific), forming alginate spheres of ~1.0×10^5 cells in 30 μl [31]. Chondrogenic media (0.1 μM dexamethasone, 50 mg/ml L-ascobate-2-phosphate, 40 mg/ml L-proline [Sigma-Aldrich], 100 μg/ml sodium pyruvate [Gibco], and 1:100 ITS+ culture supplement [BD Biosciences, San Jose, CA, http://www.bdbiosciences.com/]) in high glucose DMEM with or without indicated concentrations of hrBMP4 were replenished every 3 days. To recombine floxed *Alk2*^{CKO} cells, 1.2 nM 4-hydroxytamoxifen (Sigma-Aldrich) was added to chondrogenic media containing alginate spheres for 48 hours; genomic DNA isolated from cell pellets was amplified to confirm efficient recombination equivalent to tamoxifen treatment of monolayer culture. To assay, alginate spheres were formalinfixed for histology or incubated with 55 mM sodium citrate (Sigma-Aldrich) to release cells.

Cell Implants

A modified Matrigel implant protocol for heterotopic ossification [7, 32] was used to insert wild-type and *Alk2*$^{R206H/+}$ MEFs into the hind limbs of wild-type C57Bl/6-Tg(CAG-EGFP) 10sb/J mice (n = 4 per MEF genotype). Prior to implant, cells were labeled with Qtracker625 quantum dots (Qdots) (Invitrogen). Qdots localize to the cell cytoplasm, are unable to diffuse back out through the cell membrane, and maintain fluorescence for at least 8 weeks in vivo [33]. Labeled cells (2.67×10^6 cells per milliliter) in phenol red-free Matrigel (BD Biosciences) with 3.33 μg/ml hrBMP4 were injected (150 μl) into the right anterior tibialis muscles; contralateral left anterior tibialis muscles were injected with BMP/Matrigel (no cells). Upon injection, Matrigel solidifies into a porous scaffold that remains localized to the injection site and fully containing the cells. At 3 weeks postinjection, animals were analyzed.

MicroCT Analysis

High-resolution, cross-sectional images of injected hind limbs were obtained using a VivaCT 40 (Scanco, Nokomis, FL, http://www.scanco.com/) at a source voltage of 55 kV, a source current of 142 μA, and an isotropic voxel size of 38.0 μm. A three-dimensional (3D) image was reconstructed using Scanco microCT V6.1 software. The skeletal bone of the hind limbs and the sites of ectopic ossification were imaged separately, using two different thresholds to optimize visualization and quantification of HEO formation. The optimal threshold for the skeletal bone was a lower threshold of 212 Hounsfield and an upper threshold of 1,000 Hounsfield units. The optimal threshold for detecting ectopic ossification was a lower threshold of 150 Hounsfield and an upper threshold of 1,000 Hounsfield units. Detected ectopic mineralization was quantified using Scanco microCT V6.1 software.

Histology and Immunohistochemistry

Chondrogenic alginate spheres were formalin-fixed overnight then embedded in paraffin and sectioned serially at 7 μm. Deparaffinized sections were incubated with 55 mM sodium citrate (Sigma-Aldrich) at 37°C to remove alginate then stained with Alcian blue (pH 2.5) (Sigma-Aldrich) and counter-stained by nuclear fast red (American MasterTech, Lodi, CA, http://www.americanmastertech.com/). For type II collagen immunohistochemistry,

deparaffinized sections were treated for antigen retrieval with Proteinase K (20 μg/ml) (Roche, Indianapolis, IN, http://www.roche.com/); endogenous peroxidase activity was quenched with hydrogen peroxide (3%) and incubation with Background Buster (Innovex Biosciences, Richmond, CA, http://innvx.com/). Type II collagen primary antibody (1:2,000) (Abcam, Cambridge, MA, http://www.abcam.com/), 4°C overnight, was followed by incubated with anti-rabbit HRP-linked secondary antibody and detection with DAB, 3,3′-Diaminobenzidine, Rabbit SuperPicTure Kit (Invitrogen). Counterstain was hematoxylin (Sigma-Aldrich).

For Tg(CAG-EGFP) tissues, muscle was dissected from skeletal bone, fixed in zinc-formaldehyde (4%), decalcified in EDTA (pH 6.5) (Gibco), then transferred to sucrose (30%) (Thermo Fisher Scientific), and embedded in optimal cutting temperature (OTC) (American MasterTech) before serial sectioning at 7 μm and staining with Harris-Modified hematoxylin and eosin Y, safranin-O (American MasterTech), and alcian blue-hematoxylin-orange G [34]. For green fluorescent protein (GFP) immunohistochemistry, sections were permeabilized with Triton X-100 (0.1%) (Thermo Fisher Scientific), quenched with hydrogen peroxide (3%), and incubated with Background Buster. GFP primary antibody (1:3,000) (Abcam) at 4°C overnight, followed by anti-rabbit HRP-linked secondary antibody and DAB, Rabbit SuperPicTure Kit were used for detection. Counterstain was hematoxylin.

Data Analysis

Values are expressed as the mean ± SEM in line and bar graphs. All data are from a minimum of three independent experiments. All data are normalized to wild-type levels in the absence of ligand where relevant. Student's *t* test (two-sided, equal variance) was performed; significance was $p < .05$.

Results

BMP Signaling Is Dysregulated in *Alk2*$^{R206H/+}$ Cells

Altered BMP signaling in response to the mutant ALK2^{R206H} has been previously described in both transiently transfected mammalian cells and patient-derived cells [17, 18, 23–25]. However, such over-expression may lead to incorrect representation of the mutant receptor effects on biological processes. Furthermore, patient-derived cells (SHED and LCL) show variability in signaling levels due to varied genetic backgrounds of individual patients. To develop a stable and reproducible mesenchymal progenitor cell system, we isolated primary MEFs from *Alk2*$^{R206H/+}$ knockin mouse embryos [26], in which the mutant receptor is expressed from the endogenous *Acvr1* locus.

Both *Alk2*$^{R206H/+}$ and wild-type cells express the full repertoire of known type I and type II BMP receptors (Alk1, Alk3, Alk6, Actr2a, Actr2b, and Bmpr2) at similar levels (Fig. 1A). Gain-of-function activity of Alk2^{R206H} was confirmed by immunoblot assays for Smad1/5/8 phosphorylation (pSmad1/5/8). In the absence of exogenous BMP ligand, pSmad1/5/8 is negligible in wild-type cells, while signaling in *Alk2*$^{R206H/+}$ cells is detectable due to leaky receptor activity (Fig. 1B). BMP ligand induces rapid pSmad1/5/8 but this is further enhanced in *Alk2*$^{R206H/+}$ cells (Fig. 1B). The pSmad1/5/8 levels observed in MEFs are

comparable to those of patient-derived cells [24]. We further quantified the BMP signaling response by qRT-PCR to detect expression of specific BMP responsive transcription factors: *Id1*, *Id2*, *Id3*, and *Msx2* [35]. Without BMP ligand, increased expression of each factor was observed in *Alk2*$^{R206H/+}$ cells compared to wild-type cells (Fig. 1C). In the presence of BMP4, *Msx2* maintained increased expression relative to wild-type (Fig. 1C). Together, these results corroborate the dysregulated canonical BMP signaling in our MEF culture system that has been previously described in patient cells and over-expression systems [17, 18, 23–25, 36].

Dysregulated BMP Signaling Does Not Alter Cell Growth Characteristics

BMP signaling is reported to have both proliferative and antiproliferative effects depending on cell type and cell context [37–39]. In FOP, mesenchymal progenitor cells recruited during early phases of lesion formation, prior to endochondral ossification, undergo robust proliferation to form fibroproliferative regions that are positive for BMP2/4 [40]. We therefore investigated effects of the gain-of-function mutation on cell proliferation. MEFs display a typical fibroblast appearance, with indistinguishable morphologies between wild-type and *Alk2*$^{R206H/+}$ cells (Supporting Information Fig. S1A). Doubling times for wild-type and *Alk2*$^{R206H/+}$ MEFs, 25.4 ± 1.2 and 25.5 ± 1.3 hours, respectively, were not significantly different (Fig. 1D). Proliferation assayed by colony-forming unit-fibroblast (Supporting Information Fig. S1B) and BrdU incorporation in the absence and presence of BMP4 (Supporting Information Fig. S1C) and/or additional BMP ligands (data not shown) also showed no significant effect of the mutation on proliferation.

Alk2$^{R206H/+}$ Does Not Promote Spontaneous Chondrogenic Differentiation in the Absence of BMP Stimulation

Several reports have used MEFs as a tool to study cellular differentiation, commonly in the context of embryonic lethal genotypes. MEFs behave similarly to bone marrow-derived mesenchymal stromal cells in being plastic adherent with fibroblast-like morphology and having multipotent mesenchymal lineage potential both in vitro and in vivo [41–44].We confirmed that both wild-type and *Alk2*$^{R206H/+}$ MEFs are functionally multipotent progenitor cells through in vitro differentiation toward the adipocyte, osteoblast, and chondrocyte lineages. Differentiation in adipogenic media showed accumulation of lipid-containing vacuoles and increased adipocytespecific Fatty acid binding protein 4 (*Fabp4*) mRNA for both wild-type and *Alk2*$^{R206H/+}$ cultures (Fig. 2A). Of note, differentiation to adipocytes was less efficient compared to other lineages. Osteoblast differentiation was confirmed by staining for calcium deposition and mRNA quantification of osteoblast-specific osteocalcin (*Ocn*) (Fig. 2B). Enhanced osteogenesis of *Alk2*$^{R206H/+}$ cells agrees with results previously reported for FOP cells and the R206H Alk2 mutation [17, 18, 24, 25]. Chondrogenic differentiation in 3D alginate culture showed chondrocyte morphology with sulfated-glycosaminoglycans in the extracellular matrix and increased mRNAs for type II (*Col2α1*) and X collagen (*Col10α1*), with higher *Col2α1* levels in mutant cells (Fig. 2C).

To determine whether undifferentiated *Alk2*$^{R206H/+}$ cells are primed toward chondrogenesis, we examined early chondrogenic marker expression in the absence of chondrogenic inducers. During early stages of commitment toward chondrocytes, transcription factors

including *Nkx3.2/Bapx1* and *Sox5*, *6*, and *9* (the sox trio) increase in expression [45, 46]. Sox9, considered the master regulator of chondrogenesis, must be expressed in order for differentiation to occur [47]. Decreased expression of fibroblast markers (*Fsp1* and *Prrx1*) and increased expression of early chondrogenic markers (*Nkx3.2* and *Sox5/6/9*) would suggest that *Alk2*$^{R206H/+}$ cells are poised toward chondrogenesis, however, quantification of these markers in undifferentiated wild-type and *Alk2*$^{R206H/+}$ cells showed no significant differences (Fig. 3A). Protein levels of Fsp1 and Sox9 were also examined and were consistent with mRNA data (data not shown).

Previous studies demonstrated that over-expression of human R206H *ACVR1* in chick limb bud micromass culture induces BMP-independent chondrogenesis [17]. Using 3D chondrogenic alginate sphere cultures [31], we examined the effect of endogenous heterozygous expression of R206H Alk2 on spontaneous chondrogenesis in the absence of growth factors. We observed no spontaneous differentiation in wild-type or *Alk2*$^{R206H/+}$ cells, even after 3 weeks in chondrogenic media, and determined that addition of BMP ligand was necessary for chondrogenesis (Fig. 3B), as previously reported [43].We found variable induction of chondrogenesis by TGFβ superfamily ligands (BMP2, BMP4, BMP6, BMP7, and TGFβ3) at static dose and time (Supporting Information Fig. S2), with the most robust chondrogenesis in our culture system induced by BMP4.

Alk2$^{R206H/+}$ Accelerates BMP-Induced Chondrogenesis

To examine the sensitivity of *Alk2*$^{R206H/+}$ cells toward BMP-induced chondrogenesis, we examined responses to increasing concentrations of BMP4. Both wild-type and *Alk2*$^{R206H/+}$ cells showed a dose-dependent response, with increasing BMP4 producing greater numbers of chondrocytes detected by histological staining of sulfated-glycosaminoglycans (Fig. 4A, 4B). However, *Alk2*$^{R206H/+}$ cells showed enhanced sensitivity with a twofold increase in the number of cells differentiated to chondrocytes at low BMP4 doses; these differences between wild-type and *Alk2*$^{R206H/+}$ cultures diminished as the cultures reached maximal differentiation (Fig. 4B).

To further investigate the heightened BMP-induced chondrogenic differentiation of *Alk2*$^{R206H/+}$ cells, we quantified the progression of wild-type and *Alk2*$^{R206H/+}$ cells toward chondrogenesis over time in the presence of low-dose BMP4 (15 ng/ml). Type II collagen detection (Fig. 4C) demonstrated that *Alk2*$^{R206H/+}$ cells more rapidly achieved chondrocyte properties. Quantification of type II collagen-positive cells showed an increase in the number of chondrocytes present in *Alk2*$^{R206H/+}$ cultures compared to wild-type at days 7 and 10 (data not shown), and also indicated that wild-type differentiation levels reach those of *Alk2*$^{R206H/+}$ cells with time.

Quantified expression of early chondrocyte-specific mRNAs *Sox9*, *Col2α1*, and aggrecan (*Acan*) [48] showed a significant increase in *Sox9* and *Col2α1* mRNA in differentiating *Alk2*$^{R206H/+}$ cells compared to wild-type beginning at 7 days, while *Acan* expression increased at 10 days (Fig. 4D). These data support that the mutation affects chondrogenesis at earlier stages of differentiation and suggest that early chondrogenic stage transcript expression is prolonged by the mutation. Together, these results suggest that *Alk2*$^{R206H/+}$

MEFs differentiate to chondrocytes more rapidly and with increased production of chondrogenic transcripts and matrix proteins.

Alk2$^{R206H/+}$ Cells Contribute to and Promote HEO In Vivo

We investigated whether *Alk2*$^{R206H/+}$ cells could specifically induce HEO in vivo by implanting cells into skeletal muscle. Wild-type or *Alk2*$^{R206H/+}$ donor cells labeled with red Qdots were implanted with BMP4 (nonosteoinductive amounts; 500 ng) into wild-type host mice ubiquitously expressing GFP. After 21 days, histological sections through the implants were evaluated for tissue morphology and to determine the fate of implanted donor cells (Fig. 5A). Donor wild-type cell implants are detected as undifferentiated or fibroblast-like cells within the implant region. By contrast, *Alk2*$^{R206H/+}$ donor implants differentiated to both immature (low proteoglycans) and mature hypertrophic (high proteoglycans and anuclear) chondrocytes within the implant region. A fraction of chondrocytes retained Qdots, with which MEFs were initially labeled, indicating that implanted *Alk2*$^{R206H/+}$ donor cells directly differentiated to chondrocytes (Fig. 5A). Within wild-type cell implants, Qdots are in undifferentiated fibroblast-like cells. To determine host cell contributions to HEO, cells within the implants were probed with GFP antibody to detect GFP-tagged host cells. Regardless of wild-type or mutant donor cells, GFP-positive host cells migrated into implants. Within areas of HEO induced by *Alk2*$^{R206H/+}$ cells, both GFP-positive and GFP-negative chondrocytes were present indicating that *Alk2*$^{R206H/+}$ cells support a permissive environment for HEO and that wild-type cells are recruited to contribute to ectopic cartilage.

MicroCT demonstrated that control limbs receiving BMP4 without cells did not develop detectable mineralization (Fig. 5B). (BMP implant models for heterotopic ossification require a minimal dose of 2.5 μg BMP for consistent bone formation [7].) Limbs implanted with wild-type cells developed no measureable mineralization, with the exception of one mouse with very low levels of mineralization (animal 189), while all limbs with *Alk2*$^{R206H/+}$ cells developed robust mineralization (Fig. 5B). Quantification confirmed that significantly more mineralization occurred in the presence of implanted *Alk2*$^{R206H/+}$ cells compared to wild-type cells (Fig. 5B); this appears due to the presence of mature mineralized cartilage although bone is also present as shown by detection of type 1 collagen (Supporting Information Fig. S3). Small fragments of bone are observed neighboring regions containing hypertrophic chondrocytes (Fig. 5A).

Alk2 Expression Is Required During Initial Stages of Chondrogenesis

The accelerated chondrogenesis of *Alk2*$^{R206H/+}$ cells in vitro coupled with their induction of robust HEO in vivo suggested that enhanced BMP signaling through Alk2 contributes significantly in these cellular events; however, it remained undetermined whether these effects are downstream of general BMP signaling or dependent on signaling specifically through Alk2. Quantification of type I BMP receptor mRNA expression during chondrogenesis revealed unique transcriptional regulation patterns of each receptor during progenitor cell commitment to chondrocytes (Fig. 6A). *Alk2* mRNA was most abundant in undifferentiated MEFs and decreased rapidly upon differentiation, while *Alk3* mRNA remained relatively stable throughout and *Alk6* mRNA was most abundant in differentiated chondrocytes. The rapid and early decrease of *Alk2* mRNA suggested that Alk2 has a

specific contribution to early stage chondrogenesis and the accelerated phenotype observed in *Alk2*$^{R206H/+}$ cells.

To investigate this, primary Alk2$^{fl/fl}$;Esrl/Cre MEFs, which knockout Alk2 (*Alk2*^{CKO}) upon tamoxifen-induced Cre recombination, were assayed in vitro. *Alk2*^{CKO} cells show a twofold decrease of pSmad1/5/8 compared to wild-type cells, indicating that Alk2 contributes significantly to BMP signaling (Fig. 6B). Loss of Alk2 prior to chondrogenic induction (−48 hours) severely inhibited differentiation, with only an occasional chondrocyte observed and mRNA expression of chondrocyte markers *Sox9*, *Col2α1*, and *Acan* all significantly decreased at 14 days of culture (Fig. 6C). To identify the critical time window during which Alk2 is required, *Alk2*^{CKO} cells were deleted for Alk2 at various times prior to and during chondrogenic differentiation (Fig. 6C). Knockout of Alk2 concurrently with chondrogenic induction (0 hours) maintained a significant decrease in chondrocyte markers. However, knockout of Alk2 at >24 hours postchondrogenic induction (24 and 48 hours) showed differentiation comparable to wild-type cells (Fig. 6D). Together, these data indicate that Alk2 signaling directly modulates chondrocyte differentiation potential and support that the enhanced signaling by of Alk2^{R206H} during initial stages of chondrogenesis is sufficient to accelerate the chondrogenic program.

Discussion

FOP is a unique disorder in which one tissue (skeletal muscle, tendon, or ligament) is replaced with another—endochondral bone. Although gain-of-function ALK2 mutations are identified as the sole genetic cause of heterotopic (extraskeletal) ossification in FOP [6], current understanding of disease progression at the cellular and molecular levels is limited. It is well established that ALK2$^{R206H/+}$ progenitor cells have enhanced BMP signaling and osteogenic differentiation [17, 18, 24, 25]; however, a direct effect of the endogenous patient mutation on chondrogenic differentiation, a key process that precedes osteoblastogenesis during HEO, remained to be established. In this study, we recapitulated the heterozygous FOP patient mutation in MEFs to determine the contribution of Alk2^{R206H} in chondrogenesis which is known to precede and provide the proper environmental context for ectopic endochondral bone formation in FOP. We report that *Alk2*$^{R206H/+}$ cells have enhanced sensitivity toward chondrogenesis both in vitro and in vivo in the presence of BMP ligand, indicating a direct consequence of heightened Alk2 signaling. In vivo, Alk2$^{R206H/+}$ progenitor cells appear to play a role in establishing a HEO permissive environment, evidenced by recruitment of wild-type cells. Furthermore, we determined that signaling through Alk2 regulates early chondrogenic commitment that is not compensated by other type I BMP receptors.

Several reports have used MEFs as a tool to study cellular differentiation, commonly in the context of embryonic lethal genotypes for which bone marrow mesenchymal stem cells (MSCs) or other adult tissue-derived stem cells are not obtainable. MEFs behave similarly to bone marrow MSCs in that they are plastic adherent, express specific surface antigens, and have multipotent potential toward mesenchymal lineages in vitro and in vivo [41, 43, 44, 49–51], demonstrating that MEFs fulfill the minimal criteria for MSCs [52]. Germline transmission of knockin *Alk2*$^{R206H/+}$ is perinatal lethal [26] and harvesting MEFs as

mesenchymal progenitor cells enabled us to investigate the effects of endogenous heterozygous expression of the mutant receptor. This approach is advantageous compared to over-expression systems which may introduce artificial or exaggerated interpretations of receptor function in biological processes. We confirmed that our MEFs, as a progenitor cell model, possessed multipotent potential in vitro, and both wild-type and Alk2$^{R206H/+}$ MEFs differentiate to adipocytes, osteoblasts, and chondrocytes.

In the absence of ligand, Alk2$^{R206H/+}$ MEF progenitor cells showed mild leaky BMP pathway activation that was increased ~20% over wild-type. This finding contrasts with over-expression systems in which signaling appears at near maximum detectable capacity in the absence of ligand [17, 18, 25], but is similar to levels observed for patient-derived cells [24]. While Alk2$^{R206H/+}$ MEFs have increased BMP signaling in the absence of ligand, this enhancement was not sufficient to promote spontaneous, BMP-independent, chondrogenic differentiation as was reported in an ALK2^{R206H} over-expression system [17]. BMP signaling promotes expression of the Sox9 transcription factor in the context of chondrogenic induction [53], but we found no significant differences in *Sox9* mRNA levels between undifferentiated wild-type and *Alk2*$^{R206H/+}$ cells or for other early chondrogenic markers. Fibroblast-specific gene expression was also consistent between undifferentiated wild-type and *Alk2*$^{R206H/+}$ cells, not decreased for *Alk2*$^{R206H/+}$, further supporting that mutant cells are not precommitted. Wild-type and *Alk2*$^{R206H/+}$ cells were indistinguishable by several other analyses including cell morphology, growth rates, and BMP receptor repertoire.

By contrast, wild-type and *Alk2*$^{R206H/+}$ cells showed significant divergence when treated with BMP ligand. A clear dose effect for BMP4-induced chondrogenesis was observed for wild-type and *Alk2*$^{R206H/+}$ cells, but with increased sensitivity toward differentiation at lower concentrations for *Alk2*$^{R206H/+}$ cells. This effect is likely due to the already active BMP signaling in mutant MEFs and FOP patient-dtatic BMP4 concentration, *Alk2*$^{R206H/+}$ cells additionally show accelerated differentiation with earlier appearance of chondrocyte morphology, extracellular matrix, and increased levels of chondrocyte-specific transcripts. In a previous study designed to demonstrate ligand-independent signaling of Alk2^{R206H}, cells over-expressing the mutation in the presence of the BMP antagonist Noggin showed increased *Sox9* and *Col2α1* expression compared to wild-type Alk2 over-expression [17]. Our results show that while endogenous *Alk2*$^{R206H/+}$ expression levels are insufficient to initiate chondrogenesis, the mutant cells are primed and show a sensitized response to ligand with enhanced expression of the early chondrogenic markers *Sox9* and *Col2α1*.

We further demonstrated that *Alk2*$^{R206H/+}$ progenitor cells alone, that is in the context of a wild-type cell/tissue environment, can form endochondral extraskeletal bone tissue in vivo. As in our in vitro chondrogenesis experiments, low concentration of BMP4 was required to activate the cells. However, the concentration used is well below osteo-inductive levels (approximately fivefold less) [32] and was not sufficient to promote HO in the absence of implanted Alk2$^{R206H/+}$ cells; wild-type cell implants appear as dense undifferentiated fibroblast-like cells. Of note, BMP2/4 ligand is detected in patient lesions prior to the appearance of chondrocytes [40], suggesting that the mutation, together with endogenous BMPs, may direct lineage decisions toward cartilage.

We also observed that ectopic chondrocytes consist of not only implanted *Alk2*$^{R206H/+}$ cells but also recruited wild-type host cells. These data are consistent with the spontaneous HEO that forms in chimeric knockin *Alk2*$^{R206H/+}$ mice in which ectopic cartilage included both *Alk2*$^{R206H/+}$ mutant cells and wild-type cells [26]. These data support that Alk2$^{R206H/+}$ progenitor cells at the site of lesions not only participate in the formation of ectopic cartilage but also alter the tissue environment to support the differentiation of wild-type cells.

Alk2 mRNA levels were highest in undifferentiated cells and *Alk2* expression rapidly decreased during chondrogenic differentiation of wild-type MEFs. Previous studies on chick limbs indicated that *Alk2* mRNA expression is higher in resting and proliferating chondrocytes compared to hypertrophic chondrocytes [54]. Expression patterns in undifferentiated MEFs therefore appear to correlate with immature chondrocytes of the growth plate. Other type I BMP receptors, *Alk3* and *Alk6*, were regulated differently than *Alk2* in our cultures and align with known patterns of these receptors in the mouse growth plate [21, 55]. Alk3 and Alk6 have essential and relatively overlapping contributions to BMP signaling in the mouse growth plate with Alk3 protein most highly expressed in hypertrophic chondrocytes and Alk6 in proliferating and prehypertrophic chondrocytes [21, 55]. Little information is available for Alk1 expression patterns [21] and *Alk1* was not abundant in MEFs.

We determined that Alk2 deletion prior to or during the first 24 hours of chondrogenic induction caused substantial inhibition of BMP-induced chondrogenesis. By contrast, delaying Alk2 knockout until 24 hours after chondrogenic induction resulted in a wild-type phenotype. Interestingly, in MEFs, the remaining type I receptors Alk3 and Alk6 were not able to compensate for early loss of Alk2, indicating that signaling through Alk2 is not equivalent to signaling through Alk3 and/ or Alk6, at least in the context of chondrogenesis, and/or that Alk2 is an obligate partner in type I receptor heterodimers during early chondrogenesis [56]. Loss of Alk2 has also been demonstrated to reduce proliferation, extension, and fusion of mandibular Meckel's cartilage of Alk2/Wnt1-Cre knockout mice, where proper development of the mandible requires tight regulation of BMP signaling [57]. In agreement with this study that examined embryonic skeletal development specifically from the neural crest lineage, our data provide support for an important role for Alk2 in postnatal bone formation as well. Together these data support that Alk2 signaling is important for commitment toward chondrogenesis and that Alk2 modulates the progression of differentiation. Whether Alk2 is essential for terminal chondrogenic differentiation remains to be elucidated. In comparing the inhibited differentiation of *Alk2*^{CKO} cells with accelerated differentiation of *Alk2*$^{R206H/+}$ cells, we conclude that stimulation of Alk2^{R206H} with BMP4 within the first 24 hours results in an enhanced and potentially unique signaling mechanism to promote chondrogenesis.

Conclusions

Many outstanding questions remain including the origins of progenitor cells in lesions, how inflammation preceding chondrogenesis may influence differentiation, and understanding how Alk2 signaling during early chondrogenic induction is distinct from contributions by other type I receptors. We demonstrate for the first time that heterozygous R206H Alk2

directly impacts progenitor cell differentiation toward chondrogenesis and that this process may be mechanistically regulated by unique receptor signaling during early chondrogenic commitment, thereby clarifying a direct role for $Alk2^{R206H}$ in promoting FOP HEO and indentifying Alk2-specific BMP signaling at the onset of chondrogenesis as a therapeutic target to prevent heterotopic ossification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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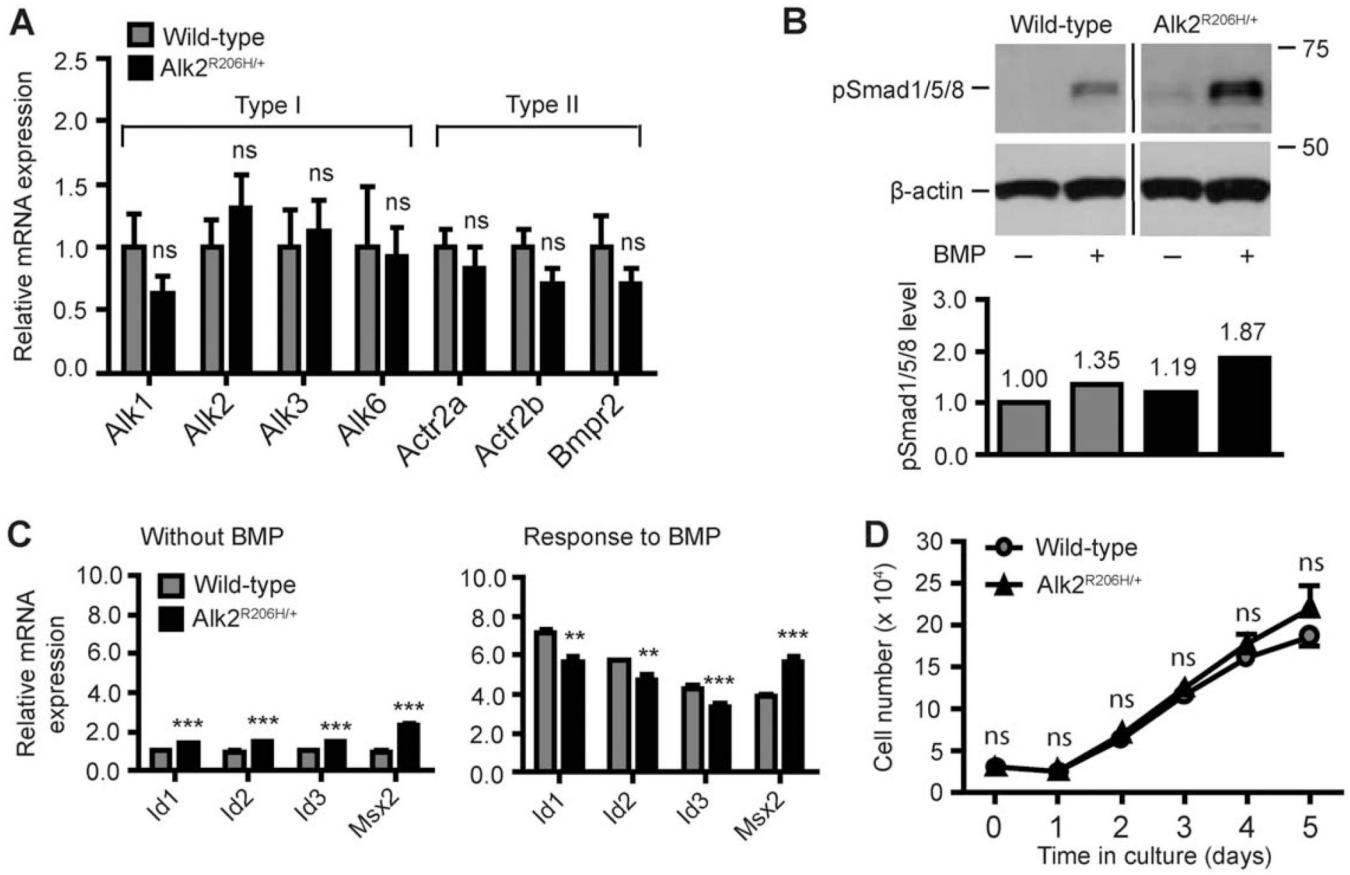

Figure 1.

Enhanced BMP signaling of *Alk2*$^{R206H/+}$ mouse embryonic fibroblasts (MEFs). **(A):** Expression of type I and type II BMP receptor mRNAs in undifferentiated MEFs was quantified and normalized to Gapdh levels. **(B):** Immunoblot detection of pSmad1/5/8 in the absence and presence of BMP4. Quantified pSmad1/5/8 was normalized to β-actin. Lanes were run on the same gel but are noncontiguous. **(C):** BMP target genes expression without BMP (left) and in response to BMP4 (15 ng/ml) for 2 hours (right) detected by qRT-PCR and normalized to Gapdh levels. **(D):** Growth curves of wild-type and *Alk2*$^{R206H/+}$ MEFs. All data represent mean ± SEM; **, $p \leq .01$; ***, $p \leq .001$; ns = not significant. Abbreviation: BMP, bone morphogenetic protein.

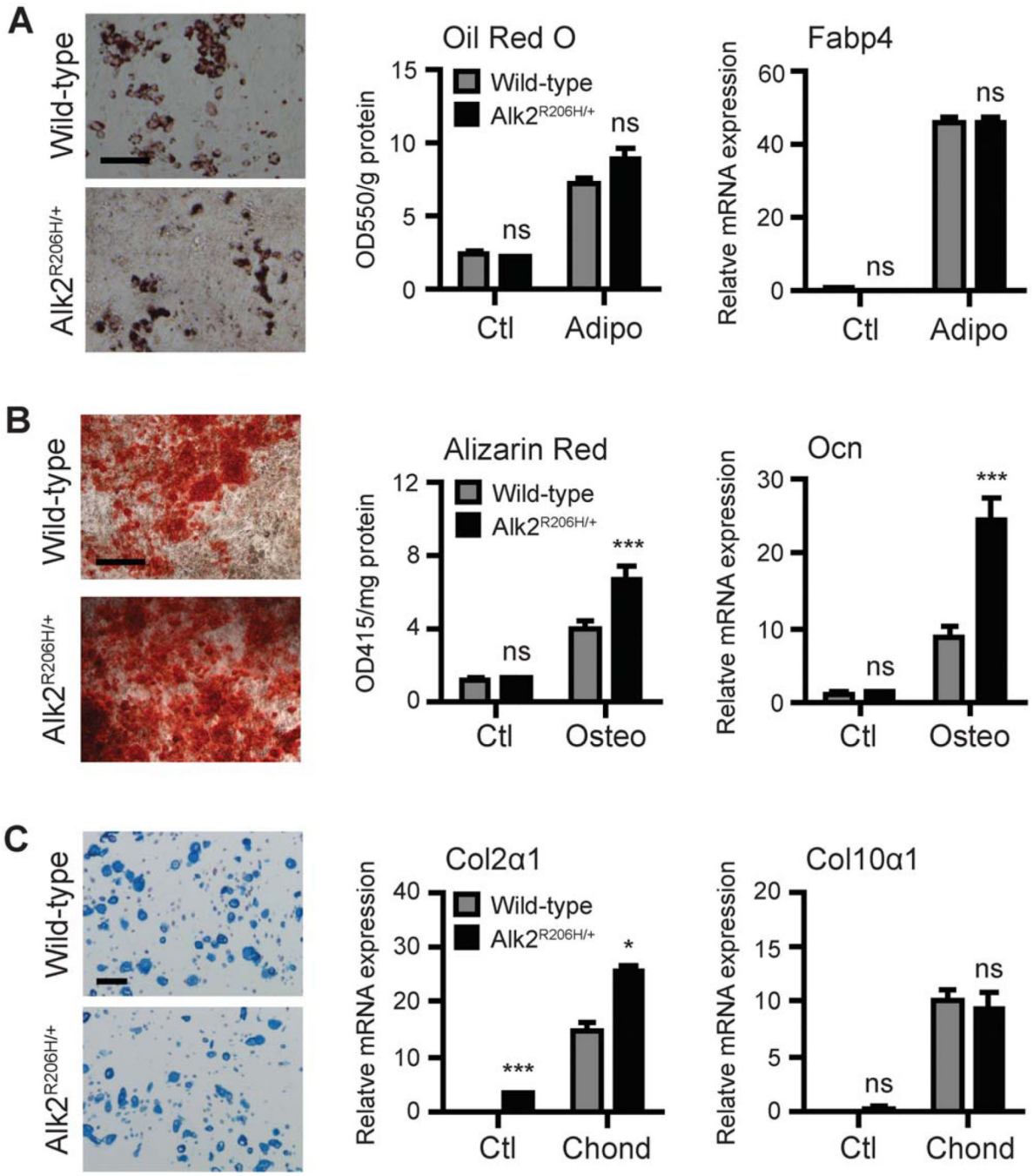

Figure 2.

Mesenchymal multipotency of mouse embryonic fibroblasts (MEFs). **(A):** MEFs in adipogenic media (14 days) were stained with oil red O to visualize lipid droplets and quantified. *Fabp4* expression was normalized to *Gapdh*. **(B):** MEFs in osteogenic media (14 days) were stained with Alizarin Red to visualize calcium deposition and quantified. *Ocn* expression was normalized to *18S* rRNA. **(C):** MEFs in chondrogenic media containing BMP4 (100 ng/ml) (14 days) were stained with Alcian blue to visualize chondrocyte morphology and matrix. *Col2α1* and *Col10α1* expressions were normalized to *18s* rRNA.

All data represent mean ± SEM; $n = 3$; *, $p \leq .05$; ***, $p \leq .001$; ns = not significant. Scale bars = 100 μm.

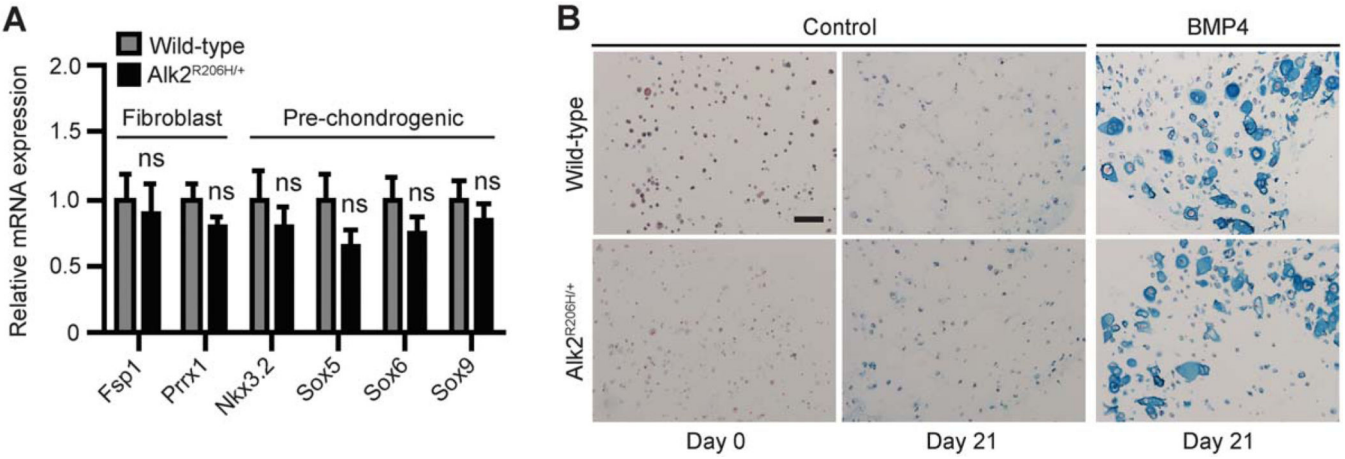

Figure 3.

Alk2$^{R206H/+}$ does not predispose cells toward chondrogenesis. **(A):** Expression of *Prrx1* and *Fsp1* (fibroblast) and *Nxk3.2* and *Sox5/6/9* (prechondrogenic) mRNAs were quantified in undifferentiated mouse embryonic fibroblasts (MEFs) and normalized to *18S* rRNA. **(B):** MEFs cultured in three-dimensional alginate spheres with chondrogenic media in the absence (control) or presence of BMP4 (100 ng/ml) for 21 days were sectioned and stained with Alcian blue to visualize chondrocyte morphology and matrix. All data represent mean ± SEM; ns = not significant.

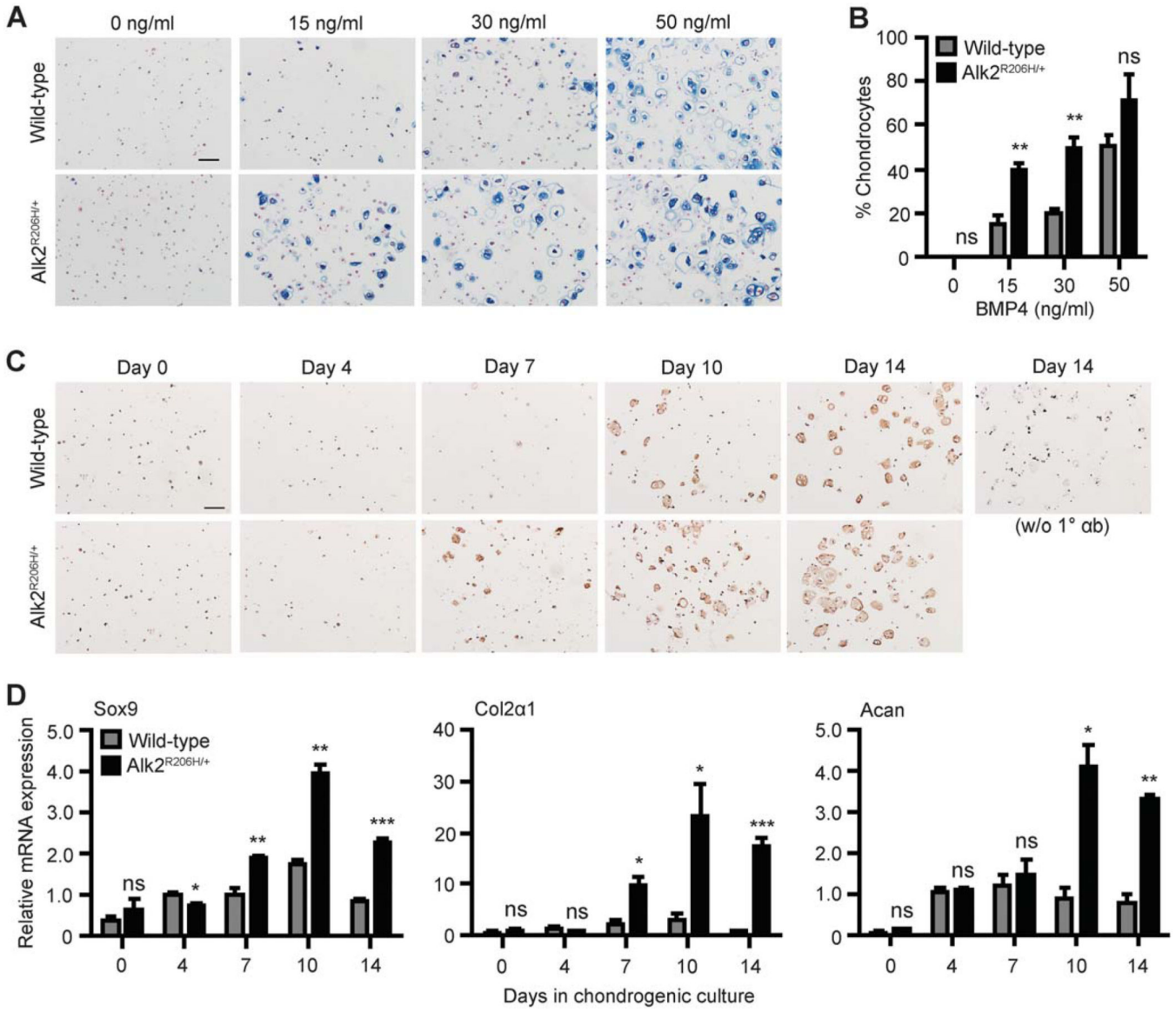

Figure 4.

Increased sensitivity and accelerated chondrogenic differentiation by *Alk2*$^{R206H/+}$. **(A):** Mouse embryonic fibroblasts (MEFs) in three-dimensional chondrogenic culture with increasing BMP4 concentrations were stained with Alcian blue (7 days). **(B):** Alcian bluepositive cells were quantified as the percentage of chondrocytes relative to total cells in field. **(C):** MEFs in chondrogenic media with 15 ng/ml BMP4 were detected for collagen type II over 14 days. Negative control is without primary antibody. **(D):** Expression of chondrocyte-specific genes over time was quantified by qRT-PCR and normalized to *18S* rRNA. All data represent mean ± SEM; *, $p \leq .05$; **, $p \leq .01$; ***, $p \leq .001$; ns = not significant. Scale bars = 100 μm.

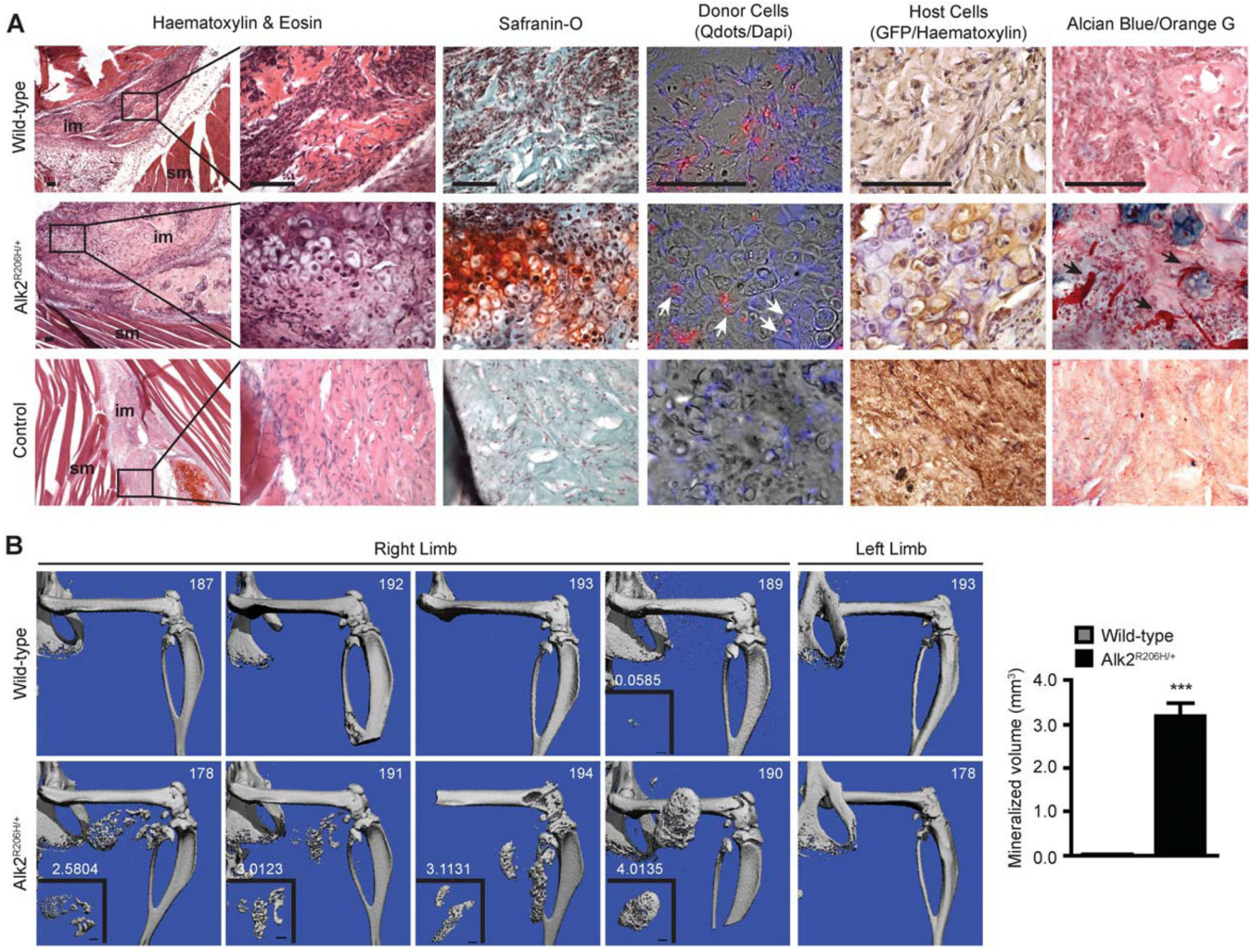

Figure 5.

Alk2$^{R206H/+}$ cells induce heterotopic endochondral ossification (HEO) in vivo. **(A):** Serial sections through implants were examined for endochondral ossification. Hematoxylin and eosin staining shows implant (im) adjacent to normal skeletal muscle (sm) tissue. Safranin-O (red) detects chondrocytes. Red fluorescent Qdots, from labeled donor cells, are present within chondrocytes of *Alk2*$^{R206H/+}$ cell implants (arrows). GFP antibody detection identifies infiltrated GFP-tagged host cells within the implants (all conditions) and a mixture of GFP^{-} (donor) and GFP^{+} (host) chondrocytes participating in HEO. Alcian blue/orange G staining differentiates cartilage (blue) from surrounding fragments of mature bone (bright pink; arrows). Scale bars = 100 μm. **(B):** Wild-type or Alk2$^{R206H/+}$ mouse embryonic fibroblasts with 500ng BMP4 were injected into mouse hind limb muscles (right); the contralateral limb (left) was injected with BMP alone and microCT evaluated ossification after 21 days. Insets highlight detected mineralization in regions of cell implants. Where ectopic mineralization was detected the total mineralized volume (mm3) was quantified using Scanco software and is shown numerically above the sample. Data represent mean ± SEM; ***, $p \leq .001$. Scale bars = 1 μm. Abbreviation: GFP, green fluorescent protein.

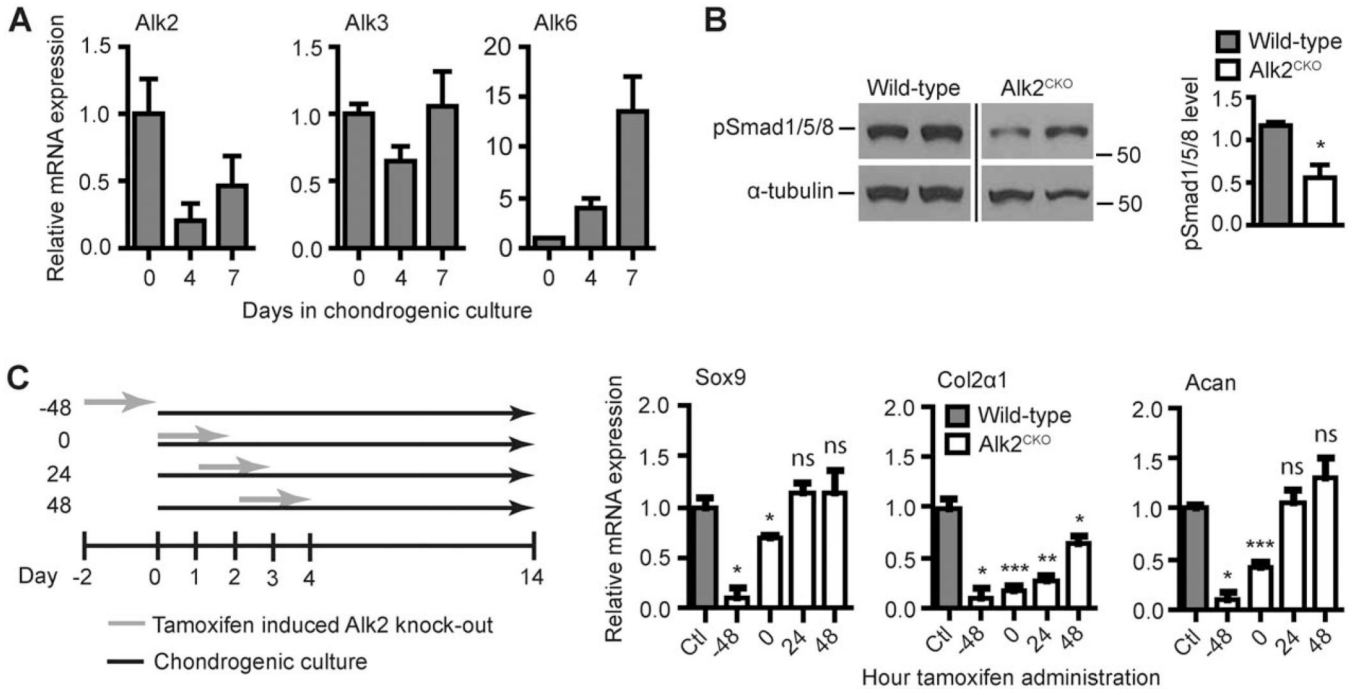

Figure 6.

Alk2 expression is required during early chondrogenesis. **(A):** Expression of *Alk2*, *Alk3*, and *Alk6* type I BMP receptors during early chondrogenic differentiation of wild-type mouse embryonic fibroblasts (MEFs) was determined by qRT-PCR and normalized to *18S* rRNA. **(B):** BMP signaling in wild-type and Alk2^{CKO} MEFs in the presence of BMP4 was detected by immunoblot analysis of pSmad1/5/8 and normalized to α-tubulin. **(C):** Schematic shows timing of Alk2 knockout (arrow) before or during chondrogenic differentiation. Chondrocyte-specific markers were assessed by qRT-PCR after 14 days and normalized to β-2-microglobulin mRNA. All data represent mean ± SEM; *, $p \leq .05$; **, $p \leq .01$; ***, $p \leq .001$; ns = not significant.