ACVR1<sup>R206H</sup> FOP mutation alters mechanosensing and tissue stiffness during heterotopic ossification

Julia Haupt<sup>a,b</sup>, Alexandra Stanley<sup>a,b</sup>, Claire M. McLeod<sup>a,c,d</sup>, Brian D. Cosgrove<sup>a,c,d</sup>, Andria L. Culbert<sup>a,b</sup>, Linda Wang<sup>a</sup>, Foteini Mourkioti<sup>a,e</sup>, Robert L. Mauck<sup>a,c,d,f</sup>, and Eileen M. Shore<sup>a,b,g,*</sup>

Departments of <sup>a</sup>Orthopaedic Surgery, <sup>b</sup>Cell and Developmental Biology, and <sup>c</sup>Genetics and <sup>d</sup>Center for Research in FOP and Related Disorders, Perelman School of Medicine, and Departments of <sup>e</sup>Bioengineering and <sup>f</sup>Mechanical Engineering and Applied Mechanics, School of Engineering and Applied Science, University of Pennsylvania, Philadelphia, PA 19104; <sup>g</sup>Translational Musculoskeletal Research Center, Corporal Michael J. Crescenz VA Medical Center, Philadelphia, PA 19104

ABSTRACT An activating bone morphogenetic proteins (BMP) type I receptor ACVR1 (<sup>A</sup>CV<sup>R</sup>1<sup>R206H</sup>) mutation enhances BMP pathway signaling and causes the rare genetic disorder of heterotopic (extraskeletal) bone formation fibrodyplasia ossificans progressiva. Heterotopic ossification frequently occurs following injury as cells aberrantly differentiate during tissue repair. Biomechanical signals from the tissue microenvironment and cellular responses to these physical cues, such as stiffness and rigidity, are important determinants of cell differentiation and are modulated by BMP signaling. We used an Acvr1<sup>R206H/+</sup> mouse model of injury-induced heterotopic ossification to examine the fibroproliferative tissue preceding heterotopic bone and identified pathologic stiffening at this stage of repair. In response to microenvironment stiffness, in vitro assays showed that Acvr1<sup>R206H/+</sup> cells inappropriately sense their environment, responding to soft substrates with a spread morphology similar to wild-type cells on stiff substrates and to cells undergoing osteoblastogenesis. Increased activation of RhoA and its downstream effectors demonstrated increased mechanosignaling. Nuclear localization of the pro-osteoblastic factor RUNX2 on soft and stiff substrates suggests a predisposition to this cell fate. Our data support that increased BMP signaling in Acvr1<sup>R206H/+</sup> cells alters the tissue microenvironment and results in misinterpretation of the tissue microenvironment through altered sensitivity to mechanical stimuli that lowers the threshold for commitment to chondro/osteogenic lineages.

INTRODUCTION

Many cancers, cardiovascular disease, and acute and chronic fibrosis are accompanied by increased extracellular matrix deposition and increased tissue stiffness (Ingber, 2003). Normal physical properties of tissues within the body have great diversity, with stiffness ranging from very soft (brain, fat tissue) to rigid (bone) (Cox and Erler, 2011). Cells interpret their environment through force sensing by pulling on surrounding matrix to measure the levels of stiffness and then respond to these physical cues in their tissue microenvironment through activation of mechanosensing signaling pathways. Signals transduced by sensing tissue stiffness impact cell fate decisions by providing instructive differentiation signals. Mechanosensing is regulated and operative during development, leading to diversity in differentiation and organogenesis/morphogenesis, and during postnatal life for maintenance of tissue homeostasis and facilitating regeneration and wound healing.
processes (Engler et al., 2006; Georges et al., 2006; Chanet and Martin, 2014). It has been suggested that a mechanosignaling stimulus alone can be sufficient to direct cell differentiation; for example, mesenchymal progenitor cells sensing an environment with the mechanical properties of nascent bone tissue will respond by undergoing osteogenic differentiation (Engler et al., 2006). Recently, it has become widely recognized that altered mechanosensing can also contribute to the pathogenesis of genetic and nongenetic diseases (Ingber, 2003; Dahl et al., 2008; Butcher et al., 2009; Knipe et al., 2015; Kai et al., 2016); however, whether heterotopic ossification (HO), a condition in which extraskelatal bone forms within soft connective tissues (McCarthy and Sundaram, 2005), could be influenced by altered force-sensing and differential interpretation of substrate stiffness remains underdetermined.

Fibrodysplasia ossificans progressiva (FOP; MIM #135100) is a rare genetic disease that forms extensive and progressive postnatal ossification within soft tissues (Shore and Kaplan, 2010). The heterotopic bone that forms within soft connective tissues in FOP is normal bone tissue by all evaluated criteria; its aberration lies in the lost regulation of cell fate determination. Mutations in the bone morphogenetic proteins (BMP) type I receptor ACVR1 (Activin A receptor, type 1; alias: ALK2) cause FOP, and all patients with a classic clinical manifestation of the disease possess a recurrent heterozygous c.617G→A (R206H) mutation (Shore et al., 2006; Kaplan et al., 2009). This single-nucleotide substitution results in overactivation of the BMP signaling pathway (Fukuda et al., 2009; Shen et al., 2009; van Dinther et al., 2010; Haupt et al., 2014). The BMP pathway is well established as promoting cartilage and bone formation (Rahman et al., 2015; Salazar et al., 2016), and the ACVR1 R206H mutation has been shown to enhance chondrogenesis and osteogenesis (Shen et al., 2009; van Dinther et al., 2010; Calbert et al., 2014; Haupt et al., 2014). Heterotopic ossification in FOP, as well as in more common nonhereditary HO (Pignolo and Foley, 2005; Shore and Kaplan, 2010), forms through progressive tissue and cellular events that culminate in bone tissue formation within skeletal muscle and other soft connective tissues; these bone-forming lesions are often initiated by tissue damage. In the absence of overt injury, new bone formation in FOP patients occurs spontaneously and episodically, interspersed by apparent periods of quiescence with no HO formation (Kaplan et al., 2008; Shore, 2012). These clinical observations support that factors in addition to the genetic mutation modulate the cellular activity of the mutant receptor to strongly influence disease progression.

Heterotopic ossification in FOP forms through a well-described series of changes within the tissue (Shore and Kaplan, 2010; Chakkalakal et al., 2012; Convente et al., 2018). In response to injury, initial steps of wound healing in FOP tissue appear to be normal, including an early immune response that leads to tissue degeneration and removal of damaged tissue. The transition from tissue degeneration to regeneration during normal wound healing is a fibroproliferative stage characterized by up-regulated extracellular matrix (ECM) production and remodeling. However, in FOP tissue this trajectory diverges when, instead of repairing and regenerating the injured tissue, progenitor cells aberrantly differentiate to cartilage and bone cells and endochondral bone is formed (Kaplan et al., 1993; Shore and Kaplan, 2010; Calbert et al., 2014; Convente et al., 2018). Alterations in the physical and mechanical properties of the tissue during this stage, and altered sensing of the tissue microenvironment by mutant progenitor cells that are primed to chondrogenetic differentiation by the underlying ACVR1 mutation, may have major, yet unrecognized, roles in promoting HO by creating a tissue microenvironment that is permissive and/or inductive for chondrogenic and osteogenic differentiation.

In this study, we examined in vivo stiffness and ECM properties of Acvr1R206H mutant tissue in response to injury to determine whether the physical/mechanical microenvironment of the tissue where HO forms is altered. Additionally, we determine whether the mutation modulates mechanosensing and mechanosignaling by investigating the ability of cells expressing the FOP mutation to properly sense and respond to the mechanical cues in their microenvironment. Our data support that both changes in the tissue microenvironment and the ability of cells to sense their environment are altered by the FOP Acvr1R206H mutation.

RESULTS

Tissue rigidity is increased in fibroproliferative areas following injury of Acvr1R206H/+ muscle

Muscle injury frequently triggers heterotopic bone formation in FOP patients, suggesting an aberrant wound healing response in the presence of the ACVR1R206H/+ mutation. Expression of Acvr1R206H/+ in a knock-in mouse model of FOP recapitulates all key clinical features of the disease including HO formation in response to muscle injury (Chakkalakal et al., 2012, 2016; Convente et al., 2018). To investigate changes in skeletal muscle tissue properties in response to injury, we treated Acvr1R206H/+ knock-in mice with cardiotoxin (Figure 1A). Cardiotoxin (CTX) leads to rapid muscle damage and muscle degradation that is accompanied by an inflammatory response; this catabolic phase is followed by the onset of an anabolic, reconstruction phase characterized by activation of muscle stem cells (e.g., satellite cells) that proliferate, differentiate, and subsequently form new muscle fibers in wild-type tissue (Couteaux et al., 1988; Charge and Rudnicki, 2004; Vignaud et al., 2005; Czerwinska et al., 2012).

To assay lesions in injured muscle from Acvr1−/− control littermates and Acvr1R206H/+ mice at the fibroproliferative stage, animals were killed at days 4 to 5 post-CTX injury (Figure 1A), at a time at which no heterotopic bone or cartilage has yet formed (Chakkalakal et al., 2012; Convente et al., 2018), as verified by H&E staining of the injured tissues. Phosphate-buffered saline (PBS) injection in control and mutant mice (Figure 1B) did not induce muscle injury and served as negative controls. Substantial degeneration of muscle tissue was found after CTX injection in both Acvr1R206H/+ mice and controls. Early stages of wound healing were accompanied by robust fibroproliferation in both mutant and control littermates (Figure 1, B and C). Tissue stiffness was quantified by measuring Young’s moduli through atomic force microscopy (AFM) (Levental et al., 2010) of consecutive nonfixed tissue cryosections (Figure 1D, left). Stiffness of healthy uninjured muscle was ~20 kPa, with no significant difference between Acvr1R206H/+ and control littermates (Figure 1D, right). Fibroproliferative regions in injured areas of control littermates showed a >3.5-fold reduction in rigidity compared with healthy muscle (black columns, Figure 1D, right), consistent with the ongoing turnover of damaged muscular tissue and initial stages of wound healing (Hinz, 2010). Lesions in control littermates were relatively soft (~6 kPa), indicating that these tissues are at an early wound healing (early fibroproliferation) stage, well before the appearance of repaired muscle fibers. However, at the comparable time point, tissue stiffness in fibroproliferative areas of injured Acvr1R206H/+ tissue was significantly elevated compared with controls, reaching a stiffness of ≥40 kPa, which is ~2-fold higher (Figure 1D, right, red bars) than healthy uninjured muscle, not unlike the pathological stiffening of tissues that is observed in fibrosis or scar tissue (~15–100 kPa) (Liu et al., 2010; Brown et al., 2013) and is a stiffness consistent with previously

Molecular Biology of the Cell
Differences in ECM composition and collagen organization contribute to stiffer Acvr1R206H/+ tissue

The biophysical properties of tissue can be altered by subtle differences in matrix composition and/or organization. During normal wound healing in muscle, fibro/adipogenic progenitors (FAPs) (Contreras et al., 2016) secrete increased amounts of various collagen isoforms, a process that is correlated with increased tissue stiffening (Hinz et al., 2012). Collagens are the major component of connective tissue ECM in mammals, forming a structural framework for cells and functioning as a storage system for morphogens and growth factors (Reddi, 2000). Collagen types I and III are the main collagens expressed by myofibroblasts during wound healing (Hinz, 2007; Hinz et al., 2012; Watsky et al., 2010; Klingberg et al., 2013). We therefore investigated whether the elevated stiffness within Acvr1R206H/+ lesions is accompanied by altered collagen content and organization within the fibroproliferative tissue.

To quantify changes in collagen production in further detail, we used mouse embryonic fibroblasts (MEFs) as an in vitro system. MEFs have properties of mesenchymal stem/progenitor cells including multipotent lineage potential (Lengner et al., 2004; Garreta et al., 2006; Saeed et al., 2012; Culbert et al., 2014). We previously demonstrated that Acvr1R206H/+ MEFs can undergo adipogenesis, chondrogenesis, and osteogenesis and that the mutation induces accelerated chondrogenesis and endochondral ossification (Culbert et al., 2014). Acvr1R206H/+ MEFs also have increased BMP-pSmad1/5/8 pathway activation, a hallmark of FOP patient-derived cells that has been recapitulated and verified in multiple systems (Billings et al., 2008; Shen et al., 2009; Culbert et al., 2014).

Total collagens produced by control and Acvr1R206H/+ MEFs were visualized and quantified by picrosirius red staining of cell monolayers and showed increased collagen deposition in Acvr1R206H/+ cells compared with control (Figure 2A). No differences in collagen type I mRNA levels were detected by quantitative reverse transcription-PCR (qRT-PCR) analysis; however, mutant cells expressed elevated collagen type II mRNA by quantitative reverse transcription-PCR (qRT-PCR) analysis; however, mutant cells expressed elevated collagen type II mRNA compared with controls (Figure 2B). Genotype III expression appeared to be delayed in Acvr1R206H/+ cells, with lower mRNA levels at earlier time points compared with controls.

To qualitatively assess collagens within control and Acvr1R206H/+ injured muscle, collagen type I, type II, and type III were detected by immunostaining at the fibroproliferative stage (Figure 3A). Counterstaining with hematoxylin or 4',6-diamidino-2-phenylindole (DAPI) was used to identify areas of intact muscle and damaged tissue. No qualitative differences were detected in collagen type I or reported AFM measurements of the osteoid microenvironment within bone (Engler et al., 2006). Intact skeletal muscle, located in close proximity to the lesions in the Acvr1R206H/+ CTX-injected group, also scaled to 20 kPa, supporting that tissue stiffening is locally restricted to fibroproliferative areas (Supplemental Figure 1). These results demonstrate that Acvr1R206H/+ preossous tissues are aberrantly stiffer and indicate that cells at sites of wound repair are actively constructing and experiencing a local microenvironment that has greater mechanical signaling properties than in control tissues.

FIGURE 1: Increased fibroproliferative tissue stiffness in response to skeletal muscle injury in Acvr1R206H/+ mice. (A) Timeline of experimental procedure. The Acvr1R206H/+ mutation was expressed in conditional Acvr1R206H/+ mice through doxycycline treatment 3 d prior to injection with cardiotoxin or PBS (uninjured control). Littermate controls were treated equivalently. (B) H&E staining of sections from PBS-injected or CTX-injured quadriceps showing areas of healthy muscle and fibroproliferation (arrow) 4 d post–injection of FOP mice or littermate controls. Scale bar represents 100 µm. (C) Enlarged images from insets in B. Scale bar: 50 µm. (D) Tissue stiffness was measured via AFM. Consecutive sections demonstrate increased rigidity of fibroproliferative areas (FP) in FOP lesions compared with healthy muscle (M). Graph represents mean ± SEM for N = 5–18 (in M: 5 [control] and 6 [FOP]; in FP: 10 [control] and 18 [FOP]) locations measured across three independently injured limbs. Significance was determined by two-way ANOVA (Bonferroni post test); *p < 0.05.
Ill deposition in either control or Acvr1\(^{R206H/+}\) tissues at this stage of lesion progression. Collagen type II, a collagen associated with cartilage matrix, is not expressed by fibroblasts (Olsen et al., 1989) and was not detected in control wounds; however, collagen type II was detected in fibroproliferative lesions of Acvr1\(^{R206H/+}\) tissue, indicating improper collagen production resulting from the mutation following injury (Figure 3B).

This modified composition of extracellular matrix produced by Acvr1\(^{R206H/+}\) cells and tissue prompted us to investigate in vivo structural differences more closely using second harmonic generation (SHG) imaging, a powerful approach for visualizing collagen organization in tissues (Chen et al., 2012). The degree of collagen organization within the tissue following injury was determined by blinded scoring; higher scores indicate a more organized SHG-positive ECM and lower scores indicate a more disorganized arrangement. A higher degree of collagen organization suggesting increased density of fibrillar collagen was found in Acvr1\(^{R206H/+}\) injured muscle at the fibroproliferative stage compared with control littermates (Figure 3, C and D), indicating advanced organization and maturation. Increased fibrillar density has been correlated with increased tissue stiffness and higher Young’s modulus (Carroll et al., 1989; Roeder et al., 2002; Raub et al., 2010), suggesting that changes in collagen organization contribute to the increased lesion stiffness in FOP. These findings suggest that altered ECM composition and organization in Acvr1\(^{R206H/+}\) lesions contribute to redirecting cell fate decision of progenitor cells into chondrogenic and/or osteogenic lineages, although a more detailed mechanism of how this altered content and assembly culminates in increased tissue stiffening remains to be elucidated.

**Mechanotransduction pathways are overactivated in Acvr1\(^{R206H/+}\) cells**

Cellular behavior of fibroblasts (e.g., differentiation, proliferation, ECM secretion) is strongly influenced by mechanical cues derived from the underlying substrata (Wells, 2008). Cells translate these physical forces from the microenvironment into biochemical signals that are intracellularly transmitted via multiple mechanoresponsive signaling pathways (Tzima, 2006). The Rho family of small GTPases activates one of the most studied mechanotransduction pathways and Rho signaling is a key regulator of the actin cytoskeleton. Rho cycles among inactive, GDP-bound and -active, and GTP-bound states, consequently regulating the activity of downstream effectors such as Rho kinase (ROCK), a critical regulator of cell contractility (Lessey et al., 2012). Basal activation of BMP signaling pathways, even in the absence of ligand, also has been shown to regulate cell contractility in mesenchymal stem cells (Heo et al., 2015, 2016).

To determine the influence of the Acvr1\(^{R206H/+}\) mutation on mechanosignaling, we first evaluated the basal activation level of the Rho/ROCK pathway in Acvr1\(^{+/+}\) control and mutant Acvr1\(^{R206H/+}\) MEFs. When cultured on tissue culture plastic, which presents a very stiff substrate (Achterberg et al., 2014), Rho activation was more robustly increased in Acvr1\(^{R206H/+}\) cells compared with controls (Figure 4A). RhoA, a small GTPase, regulates its kinase effector ROCK to influence actin filament stability and cytoskeletal organization and contractility by controlling the activity of its downstream targets cofilin and myosin light chain 2 (MLC2) (Riento and Ridley, 2003; Lessey et al., 2012; Smith et al., 2018). Cofilin affects cytoskeletal organization and dynamics by acting as a severing and disassembling protein of actin filaments; Rho/ROCK-mediated signaling phosphorylates and inactivates cofilin, thereby stabilizing actin-myosin filaments (DesMarais et al., 2005). Rho signaling through ROCK can induce contractility of the actomyosin cytoskeleton either by directly activating MLC2 through phosphorylation or indirectly by inhibiting its antagonist myosin-light-chain phosphatase (Riento and Ridley, 2003). Consistent with increased Rho activation, Acvr1\(^{R206H/+}\) cells showed elevated levels of phospho-Cofilin (Figure 4B) and
phospho-MLC2 (Figure 4C) compared with controls, confirming overactivation of this mechanotransduction pathway in mutant cells cultured on rigid substrates.

Nuclear stiffness also has been shown to scale with substrate rigidity (Swift et al., 2013) and can be measured by AFM indentation of the cell in the perinuclear region (Figure 5A), with this measurement of nuclear stiffness being a strong indicator of the overall contractile state of the cell (Swift et al., 2013). Consistent with changes in the bulk contractile state of the cells, Acvr1\(^{R206H/+}\) MEF cells on rigid substrates showed increased nuclear stiffness compared with Acvr1\(^{+/+}\) controls (Figure 5B and Supplemental Material 2). Nuclear height also decreases as a function of cytoskeletal contraction on two-dimensional culture substrates (Versaevel et al., 2012) due to the increased contractility of the perinuclear actin cytoskeleton that compacts the nucleus. We found significant flattening of Acvr1\(^{R206H/+}\) nuclei in comparison to control cells (Figure 5C, left). This nuclear flattening was rescued by reducing cellular contractility through treatment with the ROCK inhibitor Y-27632 (Y27) (Figure 5C, right), supporting that an inherently higher baseline contractility causes changes in the nuclear height/morphology of Acvr1\(^{R206H/+}\) cells, providing additional evidence of increased mechanical signaling activity in mutant cells. Taken together, these data indicate that the Rho/ROCK mechanotransduction pathway is overactivated in Acvr1\(^{R206H/+}\) cells, supporting an altered biomechanical response and misinterpretation of the substrate as a contributing mechanism to FOP pathology.

**Acvr1\(^{R206H/+}\) cells misinterpret substrate rigidity**

The above studies, conducted on tissue culture plastic, support increased activation of mechanotransduction pathways by Acvr1\(^{R206H/+}\) cells. Such stiff substrates inherently increase mechanosignaling
mined by increased cell spreading (greater cell area and less circular
shape) with increasing substrate stiffness (Figure 6, A and B). In contrast, Acvr1^{R206H/+} cells showed reduced responsiveness to substrate stiffness as evidenced by the consistently higher cell size and morphology of the mutant cells across increasing rigidity. Overall, Acvr1^{R206H/+} cells exhibited significantly greater cell areas and spreading compared with control cells; this was most pronounced for Acvr1^{R206H/+} cells on softer matrices (5 kPa) which exhibited spread areas similarly to control cells on much stiffer substrates (15 kPa). These findings indicate that, in addition to creating a stiffer tissue microenvironment in FOP lesions, Acvr1^{R206H/+} cells may also misinterpret and/or be less sensitive to the mechanical cues provided by their tissue environment during injury, inducing progenitor populations to follow abnormal lineage commitment pathways.

To investigate whether altered mechanosensing is associated with an osteogenic response in Acvr1^{R206H/+} cells on softer substrates, we examined a key pro-osteoblast factor runt related transcription factor 2 (RUNX2; alias: CBFA1) that has been shown to be essential during early commitment of mesenchymal stem cells to the osteogenic lineage (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997; Kobayashi et al., 2000). Nuclear localization of RUNX2 is critical for its ability to act as a transcription factor to induce the expression of genes required for osteogenesis such as alkaline phosphatase, osteocalcin, and osterix (Komori et al., 1997; Xiao et al., 2000; Almalki and Agrawal, 2016). Previous work has shown that matrix stiffness regulates the nuclear localization of this factor in stem cells (Yang et al., 2014; Cosgrove et al., 2016), with stiffer substrates promoting more nuclear localization.

Acvr1^{+/+} control and mutant Acvr1^{R206H/+} MEFs were cultured on PA-hydrogels of various stiffnesses for 18 h, immunostained for RUNX2 protein localization (Figure 7A), and quantified for nuclear:cytoplasmic localization ratios of RUNX2 (Figure 7B). We found that on all substrate stiffnesses tested, RUNX2 nuclear localization was higher in Acvr1^{R206H/+} compared with Acvr1^{+/+} control cells (Figure 7B). Differences were most pronounced on 10 kPa, a stiffness comparable to normal muscle tissue (Engler et al., 2006). Since heterotopic bone forms within skeletal muscle tissue in patients with FOP, the difference in RUNX2 protein localization at 10 kPa stiffness may be functionally relevant. Taken together, these data strongly support that Acvr1^{R206H/+} cells misinterpret their biophysical microenvironment, which leads to important changes in key factors that direct cellular differentiation and extracellular matrix production.

DISCUSSION

Heterotopic ossification in the rare genetic disorder FOP, as well as in more common nonhereditary HO, forms through a progressive series of tissue/cellular events that culminate in bone formation...
changes could provide the permissive conditions that support subsequent progression of aberrant cell differentiation to heterotopic bone and could provide novel targets for therapeutic intervention.

Using an in vivo model (Acvr1R206H/+ knock-in mouse) of injury-induced heterotopic ossification and AFM to directly measure the physical stiffness of the tissue, we determined that the FOP mutation results in the aberrant production of a stiff, fibrotic-like environment at an early stage of repair and prior to the production of ectopic bone by mutant tissue. Our data suggest that Acvr1R206H+ cells involved in tissue repair establish a microenvironment that favors heterotopic bone formation.

ECM organization and composition, notably of collagens, are key determinants of tissue stiffness, and we determined that the fibroproliferative tissue in FOP lesions was organized to a greater extent than tissue in control lesions. Type I and type III collagens were detected at similar levels in Acvr1R206H/+ and Acvr1+/+ control lesions. However, type II collagen, which is usually restricted to specific tissues such as cartilage and is an established secondary target gene of the BMP signaling pathway (Olsen et al., 1989; Bell et al., 1997; Ng et al., 1997) was increased only in mutant repair tissue, consistent with overactivation of the BMP signaling pathway by the Acvr1R206H+ mutation. In cartilage, the type II collagen network traps negatively charged proteoglycan aggregates that imbibe water into the tissue generating compressive stiffness (Burg et al., 1996; Majd et al., 2014) and therefore type II collagen in Acvr1R206H+ lesions may also cause increased stiffening in part by increasing hydration/swelling of lesion tissue and/or improper accumulation of proteoglycans.

Cells interpret the biophysical properties of their surroundings via cell surface mechanoreceptors that are coupled to the contractile cytoskeleton and signal through several direct and indirect signaling pathways that direct cell fate through modification of chromatin organization and gene expression (Armsdorff et al., 2009; Swift et al., 2013; Smith et al., 2017, 2018). We determined that the Rho/ROCK mechanosignaling pathway, which mediates signals from the extracellular matrix, integrins, and focal adhesions that affect cellular morphology, migration, contractility, and adhesion to surface substrates (Lessey et al., 2012), is overactivated in cells with the Acvr1R206H+ mutation and that mutant cells additionally show increased nuclear and cellular stiffness, supporting that they do not correctly sense and interpret physical/mechanical cues from their tissue microenvironment. RhoA also facilitates phosphorylation of Smad1/5/8 (Derynck and Zhang, 2003; Du et al., 2011; Wang et al., 2012; Kopf et al., 2014), creating a further connection between RhoA activation and BMP signaling, two critical pathways that impact cell fate. Given the association between RhoA and osteogenic commitment (Armsdorff et al., 2009), our data suggest that RhoA and BMP pathways might act synergistically to contribute to
chondrogenic and osteogenic differentiation and endochondral ossification in FOP.

Acvr1^{R206H/+} cells mesenchymal progenitor cells incorrectly sensed and interpreted substrate stiffness, responding with a considerably spread morphology on even very soft substrates. Strikingly, this response to the physical/mechanical environment was accompanied by increased nuclear localization of RUNX2, a chondro/osteogenic transcription factor (Komori et al., 1997; Xiao et al., 1997; Matsubara et al., 2000; Wang et al., 2005; Chen et al., 2014; Almalki and Agrawal, 2016). BMPs induce expression of RUNX2 (Hay et al., 2001; Matsubara et al., 2008; Sun et al., 2015) and RUNX2 nuclear localization is positively affected by increased mechanical force (Zhang et al., 2012; Yang et al., 2014; Jazayeri et al., 2015; Cosgrove et al., 2016), supporting that increased BMP-$p$Smad1/5/8 signaling by the mutant Acvr1^{R206H} receptor together with misinterpretation of microenvironment contribute to aberrant chondro/osteogenic differentiation by Acvr1^{R206H/+} cells.

This study identifies for the first time that the FOP mutation alters cell mechanical state and mechanobiology, as well as the material properties of the wound microenvironment. We found that FOP cells are less sensitive to changing physical/mechanical features than control cells and that FOP tissue healing causes wounded muscle tissue to repair in a manner that is stiffer and more organized than normally occurs during skeletal muscle injury. These data support that the physical tissue microenvironment in which heterotopic ossification forms is both altered by cells with the ACVR1^{R206H} mutation and is differentially perceived by cells with the ACVR1^{R206H} mutation. We additionally propose that initial small-scale changes in the ECM (such as collagen type II production) act to prime formation of a stiffer tissue microenvironment. That is, BMP pathway signaling by the Acvr1^{R206H} mutation alters the repair trajectory of injured tissue, with cells interpreting softer matrices as stiffer. As tissue resident mesenchymal cells, such as the FAPs that have recently been identified as a major source of HO forming cells in a mouse model of FOP (Lees-Shepard et al., 2018), begin to respond to the stiffer environment in the context of enhanced BMP pathway signaling, a reinforcing feedback loop results. This furthers ECM production and tissue stiffening (a situation that alone is pathologic, such as in fibrosis), but in the context of increased BMP signaling and altered biomechanical sensing, the cells are directed to differentiate to form heterotopic bone.

A novel treatment strategy to prevent HO formation in FOP patients, and potentially more common, nonhereditary forms of HO, may reside in the regulation of mechanotransduction pathways during tissue repair (e.g., Fasudil [selective ROCK inhibitor], Cethrin [Rho inhibitor]) (Shimizu et al., 2001; Liao et al., 2007; Fehlings et al., 2011; Bai et al., 2013; Zhou et al., 2013). Our findings of biomechanical cues are important to consider in light of a recent therapeutic approach that has been successfully used in animal models to ameliorate fibrosis and also suggested to treat solid tumors that show increased stiffness and mechanical properties (Jain et al., 2014; Kai et al., 2016). Taken together, our data reveal the importance of mechanobiological signaling in FOP cells and provide the first direct experimental evidence that FOP progression may be influenced by the tissue microenvironment. Further studies will investigate the dependence on an altered physical environment to support heterotopic ossification, altered mechanosensing by progenitor cells, and the effects of inhibitors of biomechanical effectors on chondro/osteogenic differentiation in Acvr1^{R206H/+} cells.

MATERIALS AND METHODS

Mouse strain and animal care and use

Cells (MEFs) for in vitro experiments were obtained from Acvr1^{R206H/+} knock-in mice described in Chakkalakal et al. (2012).

Tissues for in vivo studies were obtained from Acvr1^{R206H/+}FLEx conditional-on knock-in mice (Hatsell et al., 2015; Chakkalakal et al., 2016) that were bred to include R26-rtTA and tetO-Cre as described (Chakkalakal et al., 2016; Convente et al., 2018). Here we refer to these mice as Acvr1^{R206H/+}. Acvr1^{+/+} controls were littermates that did not contain an Acvr1^{R206H} allele (as verified via PCR genotyping). To induce recombination and global expression of the mutant allele,

**FIGURE 6:** Acvr1^{R206H/+} cells misinterpret substrate elasticity. (A) Immortalized control and Acvr1^{R206H/+} (FOP) cells on gels of various rigidities (5, 10, 15, and 55 kPa) were stained with phalloidin and DAPI. (B) Cell area and aspect ratio (AR) were measured as a function of matrix elasticity. Graphs represent mean ± SEM of >350 cells from four independent experiments. Significance was determined by two-way ANOVA (comparison between genotypes; Tukey–Kramer adjustment; *p < 0.05, ***p < 0.001, NS = not significant) or one-way ANOVA (comparison between substrate stiffness; ***p < 0.001; Bonferroni’s post hoc). Scale bar represents 100 µm.
4-wk-old mice were provided a doxycycline diet (either 200 or 625 mg/kg) for at least three consecutive days. Cre-mediated recombination was verified by PCR. Littermate Acvr1<sup>+/+</sup> controls were also treated with doxycycline. Mice were not assigned to treatment groups by gender in our experiments.

**Induction of lesions in muscle tissue**

Mouse quadriceps muscles were injected with 100 μl CTX solution (10 μM in phosphate-buffered saline [PBS]; Calbiochem). Muscle injury by CTX injection is a standard experimental approach in muscle regeneration studies (Couteaux et al., 1988; Charge and Rudnicki, 2004; Vignaud et al., 2005; Czerwinska et al., 2012). PBS injection in Acvr1<sup>R206H/+</sup> and Acvr1<sup>+/+</sup> littersmates served as controls. This approach for injury induction of HO has been well characterized (Chakkalakal et al., 2016; Convette et al., 2018), with heterotopic bone forming at 10–14 d and robust fibroproliferation at 4–5 d. Animals were killed at the fibroproliferative stage to isolate lesion tissue and surrounding skeletal muscle. Tissues were embedded in optimal cutting temperature media (OCT; CryoPrep; American MasterTech Scientific) and serially sectioned at 20-μm thickness for AFM analysis or 10 μm for histology and immunohistochemistry.

**Histology and collagen immunohistochemistry**

Tissues were fixed in 4% paraformaldehyde (PFA) in PBS (Fisher Scientific), sectioned to 10 μm, and stained with Harris modified hematoxylin and eosin Y (H&E) or used for immunohistochemistry.

For collagen detection, endogenous peroxidases were quenched by incubation with 3% hydrogen peroxidase solution. Sections were blocked (Background Buster; American MasterTech) and incubated overnight at 4°C with primary antibody against collagen type I and II (Abcam; catalogue ab34710 and ab21291, respectively), collagen type III (Thermo Scientific; catalogue PAS-27828), or IgG control (Cell Signaling Technology; catalogue 3900). After incubation with HRP-linked secondary anti-rabbit antibody (Cell Signaling Technology; catalogue 7074), signal was visualized by using 3,3′-diaminobenzidine (DAB) substrate (SuperFicTure Polymer; Invitrogen). Sections were counterstained by short incubation with hematoxylin (Vector Laboratories) or by DAPI using Fluoromount-G (SouthernBioTech). Imaging was carried out using an Eclipse 90i microscope (Nikon).

**Analysis of tissue and cellular stiffness using atomic force microscopy**

Tissue and nuclear stiffness were measured via nanoindentation using an atomic force microscope equipped for simultaneous optical imaging (Agilent ILM 6000 mounted on a Nikon inverted microscope) (Roduit et al., 2009; Thomas et al., 2013; Heo et al., 2019). Z-depth imaging was performed using glass micropipettes (1-μm spherical) via nanoindentation using an atomic force microscope (Eclipse 90i microscope, Nikon). Imaging was carried out using an Eclipse 90i microscope (Nikon).

For collagen detection, endogenous peroxidases were quenched by incubation with 3% hydrogen peroxidase solution. Sections were blocked (Background Buster; American MasterTech) and incubated overnight at 4°C with primary antibody against collagen type I and II (Abcam; catalogue ab34710 and ab21291, respectively), collagen type III (Thermo Scientific; catalogue PAS-27828), or IgG control (Cell Signaling Technology; catalogue 3900). After incubation with HRP-linked secondary anti-rabbit antibody (Cell Signaling Technology; catalogue 7074), signal was visualized by using 3,3′-diaminobenzidine (DAB) substrate (SuperFicTure Polymer; Invitrogen). Sections were counterstained by short incubation with hematoxylin (Vector Laboratories) or by DAPI using Fluoromount-G (SouthernBioTech). Imaging was carried out using an Eclipse 90i microscope (Nikon).
**Second-harmonic generation**

To visualize collagen organization, SHG imaging (Han et al., 2016) of unstained, unfixed 20-μm cryosections was performed using an excitation wavelength of 800 nm (Zeiss LSM 510 with tunable Ti-Sapphire laser). Signal collected between 390 and 465 nm was considered to be SHG specific, while signal collected between 500 and 550 nm was considered to be nonspecific tissue autofluorescence and used as a control. To semiquantitatively assess SHG images (n = 7–8 per genotype), four scorers blinded to genotype scored images on a categorical scale from highly organized (score = 5) to completely disorganized (score = 1). For each image, scores were averaged and considered as a single data point that is shown in the Figure 3D boxplot; genotype score was compared using a Student’s t test.

**Preparation of polyacrylamide hydrogels**

Rigidity of native tissues was mimicked using an adjustable PA hydrogel system. PA hydrogels were prepared as described (Aratyn-Schaus et al., 2010). Elastic moduli of the gels were verified through AFM force spectroscopy. Fibronectin (20 mg/ml) coating of gel surfaces was accomplished after treatment with 2 mg/ml sulfo-SANPAH (No. 22589; Pierce Protein Biology/Life Technologies, Rockford, IL) as described previously (Aratyn-Schaus et al., 2010).

**Cell culture, mouse embryonic fibroblasts, and in vitro assays**

Knock-in Acvr1<sup>R206H/+</sup> MEFs were isolated at embryonic day 13.5 (E13.5) as previously described (Culbert et al., 2014) from mice described in Chakkalakal et al. (2012) and from wild-type Acvr1<sup>+/+</sup> mice. Cells were immortalized (Capital Biosciences, Rockville, MD) by transducing cells with recombinant lentivirus expressing SV40 large T antigen. Expression of SV40 T antigen was confirmed via transduction of 5×10<sup>6</sup> immortalized cells. Cells were confirmed by DNA sequencing. Cells were cultured in high glucose DMEM (Life Technologies) containing 10% fetal calf serum (FCS; Invitrogen). Testing for mycoplasma contamination was done after immortalization and repeated at the beginning of cultivation.

For nuclear height measurement, iMEFs were cultured in a standard growth medium (HG-DMEM, 10% FCS, 1% penicillin/streptomycin/fungiezone [PSF]), before treatment with ROCK inhibitor Y-27632 (Calbiochem; catalogue 688000) at 10 μM for 1 h. Following treatment, cells were fixed with 4% PFA, permeabilized with TX-100, and stained with DAPI. Nuclei (15–20 cells per condition) were imaged using a 100× 1.45 NA objective with a 1.7x zoom (0.07 μm/px) and a pinhole of 0.15 μm (0.5 AU) on a Nikon A1R Confocal Microscope. Measurements of nuclear height were determined from maximum projection XZ sections using the ImageJ software suite.

For mechanosensing assays, subconfluent iMEFs were seeded onto freshly prepared PA-hydrogels (described above) at a concentration of 5×10<sup>5</sup> cells/cm<sup>2</sup>. After 16 h, cells on hydrogels were fixed in 4% paraformaldehyde/PBS (Affymetrix), followed by incubation with Alexa Fluor 488 Phalloidin (Invitrogen; catalogue A12379) to visualize actin cytoskeleton and DAPI for nucleus staining. Cell morphology was analyzed in ImageJ.

**Western blotting**

Cells were lysed in buffer (RIPA; Sigma-Aldrich) supplemented with Halt Protease and Halt Phosphatase Inhibitor Cocktails (Pierce), cleared by centrifugation and quantified using BCA Protein Kit (Thermo Scientific). Proteins were electrophoresed through 10% Tris–glycine gels (Invitrogen) and transferred onto a nitrocellulose membrane (Invitrogen). Membranes were blocked in 5% milk in Tris-buffered saline (TBS) and incubated overnight at 4°C with primary antibody for cofilin, phospho-Cofilin, and β-actin (all Cell Signaling Technology; catalogue 5175, 3313, and 4967, respectively) followed by detection using a HRP-conjugated secondary antibody (Cell Signaling Technology; catalogue 7074). Membranes were incubated with HRP substrate (LI-COR) and chemiluminescence was detected and quantified with a C-DiGit blot scanner (LI-COR). Phospho- and total cofilin values were first normalized to β-actin to correct for variation in protein loading, followed by normalization of phospho- to total cofilin and, finally, to Acvr1<sup>1/2</sup> control cells.

**Rho activity assay**

Cells were cultured to confluence in 15-cm plates to obtain sufficient protein amounts for active Rho pull down. Cell lysis and pull down of active Rho used 500 μg of total protein following manufacturer's instructions (Active Rho Pull-Down and Detection Kit; Thermo Scientific; catalogue 16116). Proteins were electrophoresed through 10% Tris–glycine gels (Invitrogen), transferred onto nitrocellulose membranes (Invitrogen) and detected with anti-Rho antibody. Additional blots for total Rho and β-actin were run for all samples as loading controls.

**RNA isolation and real-time RT-PCR analysis**

RNA was isolated from MEF monolayers using TRizol (Invitrogen). Genomic DNA was eliminated from RNA samples by digestion with RNase-free DNase (Promega). RNA concentration was determined by NanoDrop and equivalent amounts for each sample were used for cDNA synthesis using High Capacity RNA-to-cDNA reagents (Applied Biosystems). Quantitative PCR analysis was performed to detect expression of collagen type I alpha 1 chain (Col1α1), collagen type II alpha 1 chain (Col2α1), and collagen type III alpha 1 chain (Col3α1) in a 7500 thermal cycler (Applied Biosystems) using forward and reverse primers (Col1α1 forward 5′-GACATGG-CAAGAAGACATCC-3′; Col1α1 reverse 5′-CTGCGGTTTTCCAC-GTCC-3′; Col2α1 forward 5′-AGAACAGATCGCTACTGG-3′; Col2α1 reverse 5′-CTTGCCCCACCTACGATGT-3′; Col3α1 forward 5′-TCAAGTCCGTAGGGGAGG-3′; Col3α1 reverse 5′-TGGTGATGGCAGAAGACCA-3′) and Fast SYBR Green PCR Master Mix (Applied Biosystems). Each sample was run in triplicate and
normalized to GAPDH followed by normalization to respective collagen content of Actr1/α on day 1.

Statistical analysis Results are presented as the mean ± SEM. Paired or unpaired data sets were analyzed using two-tailed Student’s t test or analysis of variance (ANOVA) (Bonferroni or Tukey–Kramer post-hoc test; one-way or two-way) to determine significance. Differences were considered statistically significant at p < 0.05. Significance and sample size (n for number in group or N for total sample size) are shown for each data set in the figure legends.

Study approval All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

ACKNOWLEDGMENTS This work was supported by a National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) Building Interdisciplinary Research Teams (BIRT) Award (E.M.S. and R.L.M.) from the National Institutes of Health (NIH) (3R01-AR041916-15S1). Additional support was provided by NIH Grants R01-AR071399 (E.M.S./R.L.M.) and R01-EB008722 (R.L.M.), the International Fibrodyplasia Osificans Progressiva Association, the Center for Research in FOP and Related Disorders, the Ian Calli Endowment for FOP Research, the Whitney Weldon Endowment for FOP Research, the Cali-Weldon Professorship of FOP Research (E.M.S.), NIH NIAMS F31 AR069982-01A1 (A.S.), and National Science Foundation Graduate Research Fellowship DGE-08222 (C.M.M.). Histological and mechanical testing core facility resources were through the Penn Center for Musculoskeletal Disorders (P30 AR069619). We thank Deyu Zhang, Salim Maidment, and Michael Convente for technical support for in vivo experiments. Additionally, we thank members of the “FOP laboratory” and the “Mauck laboratory” at the McKay Orthopaedic Research Laboratory for valuable comments and discussion.

REFERENCES


Almalki SG, Agrawal DK (2016). Key transcription factors in the differentiation of mesenchymal stem cells. Differentiation 92, 41–51.


phenotype to bone marrow stromal stem cells (BMSC). Stem Cell Res 8, 318–328.