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Smad1/5 and Smad4 Expression Are Important for Osteoclast Differentiation

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ABSTRACT

To investigate the necessity of the canonical BMP pathway during osteoclast differentiation, we created osteoclasts with a conditional gene deletion for *Smad1* and *Smad5* (SMAD1/5), or *Smad4* using adenovirus expressing CRE recombinase (Ad-CRE). Reduction of either Smad4 or Smad1/5 expression resulted in fewer and smaller multinuclear cells compared to control cells. We also detected changes in osteoclast enriched genes, demonstrated by decreased *Dc-stamp* and *cathepsin K* expression in both Smad4 and Smad1/5 Ad–CRE osteoclasts, and changes in *c-fos* and *Nfatc1* expression in only Smad4 Ad–CRE cells. Lastly we also detected a significant decrease in resorption pits and area resorbed in both the Smad4 and Smad1/5 Ad-CRE osteoclasts. Because we inhibited osteoclast differentiation with loss of either Smad4 or Smad1/5 expression, we assessed whether BMPs affected osteoclast activity in addition to BMP's effects on differentiation. Therefore, we treated mature osteoclasts with BMP2 or with dorsomorphin, a chemical inhibitor that selectively suppresses canonical BMP signaling. We demonstrated that BMP2 stimulated resorption in mature osteoclasts whereas treatment with dorsomorphin blocks osteoclast resorption. These results indicate that the BMP canonical signaling pathway is important for osteoclast differentiation and activity. J. Cell. Biochem. 116: 1350–1360, 2015.

KEY WORDS: BMPs; TGF-β; OSTEOCLASTS; SMADs; RESORPTION; FUSION

Bone is a living tissue that requires a balance of bone formation by osteoblasts and bone resorption by osteoclasts to maintain its integrity throughout life [Henriksen et al., 2009]. Osteoclasts are hematopoietic in origin, derived from the monocyte/macrophage lineage [Väänänen and Laitala-Leinonen, 2008]. They are large multinucleated cells formed from the fusion of multiple mononuclear precursors [Väänänen and Laitala-Leinonen, 2008]. Osteoclasts, as the primary resorptive cells of the skeleton, facilitate the removal of old bone and aid in maintaining mineral homeostasis [Boyle et al., 2003]. Numerous cytokines and growth factors govern osteoclast differentiation and activity through both autocrine and paracrine mechanisms. The most critical cytokines for promoting osteoclast differentiation are macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL). The primary sources of M-CSF and RANKL for initiating osteoclast differentiation are either osteoblasts or osteocytes, depending on the developmental stage of the animal [Zhao et al., 2002; Nakashima et al., 2011; Xiong et al., 2011].

Bone morphogenetic proteins (BMPs) are multi-functional growth factors involved in numerous signaling pathways and molecular cascades. BMPs comprise a subgroup of the transforming growth factor-beta (TGF-β) superfamily [Wozney et al., 1988]. BMP binding to its receptors induces a heterotetrameric complex of BMP type I and type II receptors. The type I receptors recognize the receptor activated

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Smads (R-Smads), which for BMP signaling are Smad1, 5 and 8. Smad4 is a common partner Smad (co-Smad) and is involved in both BMP and TGF-β/activin signaling pathways. Phosphorylation of R-Smads by the activated BMP receptor complex induces formation of a heterodimeric complex between the phosphorylated R-SMADs and co-Smads, which translocates to the nucleus. This SMAD complex regulates the transcription of target genes in conjunction with various transcription factors.

As reviewed by Giannoudis et al. [2007] the role of BMPs in skeletal homeostasis involves both osteoblasts and osteoclasts. Our lab's previously published work demonstrated that in a mouse model with deletion of a BMP inhibitor, *twisted gastrulation* (*Twsg1*), the mice exhibited increased BMP signaling and osteopenia due to increased number, size, and activity of osteoclasts [Rodriguez et al., 2009]. In subsequent experiments, we demonstrated that exogenous BMP2 directly enhances RANK Ligand (RANKL)-mediated formation of wild-type bone marrow derived osteoclasts [Jensen et al., 2010]. We further confirmed that osteoclasts express BMP receptors [Jensen et al., 2010], and in addition to others, our lab demonstrated that osteoclasts express BMP ligands 2, 4, 6 and 7 [Garimella et al., 2008; Jensen et al., 2010]. However, whether BMP ligands regulate osteoclasts in an autocrine or paracrine manner is still an area that needs to be investigated. More recently we demonstrated that loss of expression of BMP receptor type II in osteoclasts results in an osteopetrotic phenotype primarily due to changes in noncanonical BMP signaling [Broege et al., 2013]. In that same study, using the inhibitor dorsomorphin, a SMAD inhibitor, we were able to demonstrate the importance of activation of Smad1/5/8 around the time of fusion during osteoclast differentiation which is the same time that phosphorylated Smad1/5/8 is first detectable in osteoclasts [Broege et al., 2013]. To further verify the importance of canonical BMP signaling in osteoclast differentiation and ensure that dorsomorphin's effects on osteoclasts were due to inhibited BMP signaling rather than due to off-target effects, we chose to make use of a genetic approach to determine the importance of the canonical BMP pathway during osteoclast differentiation. The goal of the current study is to conditionally delete *Smad1* and *Smad5* (*Smad1/5*), or *Smad4* using CRE and control adenoviral vectors to further characterize the function of Smad signaling during osteoclastogenesis. We anticipate the results will further our understanding of the mechanisms by which BMPs regulate osteoclast differentiation and activity.

MATERIAL AND METHODS

BREEDING OF SMAD1/5^{FLFL} AND SMAD4$^{F/FLFL}$ MICE

Smad1/5 floxed mice obtained from Dr. Stephanie Pangas, Baylor College of Medicine, Houston, TX with permission obtained from Dr. Elizabeth Robertson (Oxford University, United Kingdom) and Dr. An Zwijsen (VIB and Center for Human Genetics, KU Leuven, Belgium) who generated the *Smad1*$^{fl/fl}$ and *Smad5*$^{fl/fl}$ mice, respectively in a mixed background of C57Bl/6 and 129SV as described in [Huang et al., 2002; Umans et al., 2003]. *Smad4* floxed mice were created by Dr. Chuxia Deng [Yang et al., 2002] and were provided to us by Dr. Michael O'Connor (University of Minnesota). Mice were in a C57Bl/6 background. The use and care of these mice was reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee.

HARVESTING OF BONE MARROW/PRIMARY OCLS

Primary bone marrow macrophages were harvested from the femurs and tibiae of 4-week-old *Smad1/5* floxed or *Smad4* floxed mice. The femurs and tibiae were dissected and adherent tissue was removed. The ends of the bones were cut and the marrow was flushed from the inner compartments. Red blood cells (RBC) were lysed from the flushed bone marrow tissue with RBC lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4) and the remaining cells were plated on 100 mm plates and cultured overnight in osteoclast medium [phenol red-free Alpha-MEM (Gibco) with 5% heat-inactivated fetal bovine serum (Hyclone), 25 units/mL penicillin/streptomycin (Invitrogen), 400 mM L-Glutamine (Invitrogen), and supplemented with 1% CMG 14-12 culture supernatant containing M-CSF]. The non-adherent cell population, including osteoclast precursor cells, was then carefully separated and re-plated at approximately 1.7×10^4 cells/cm^2 in a 12 well plate with osteoclast medium supplemented with 1% CMG 14-12 culture supernatant. Two days later, this medium was replaced with medium containing 1% CMG 14-12 culture supernatant and 30 ng/mL RANKL (R&D Systems) to stimulate osteoclast differentiation. For osteoclast resorption assays, experiments were performed and quantitated using calcium phosphate plates (Corning).

ADENOVIRAL TRANSFECTION

Bone marrow macrophages were isolated as described above. Prior to stimulation with RANKL, the cells were incubated with 100 MOI of adenovirus (EGFP or CRE expressing) for 3 h at 37°C in the presence of M-CSF. After 3 h, medium containing the adenovirus was removed and cells were fed with 1% CMG 14-12 culture supernatant and RANKL (30 ng/mL). After 5 days, RNA was extracted for use in real-time RT-PCR, protein was extracted for Western blotting, or cells were stained for TRAP.

HARVESTING RNA

Quantitative real-time PCR was performed using the MyiQ Single Color Real-Time PCR Detection System (Biorad). RNA was harvested from cells using Trizol Reagent (Ambion, Life Technologies) and quantified using UV spectroscopy. cDNA was prepared from 1 μg RNA using the iScript cDNA Synthesis Kit (Biorad) as per the manufacturer's protocol. Experimental genes were normalized to *L4*. c-*fos* (Forward) 5′-CCA AGC GGA GAC AGA TCA ACT T (Reverse) 5′-TCC AGT TTT TCC TTC TCT TTC AGC AGA; *NFATc1* (Forward) 5′-TCA TCC TGT CCA ACA CCAAA; (Reverse) 5′-TCA CCC TGG TGT TCT TCC TC; *Cathepsin K* (Forward) 5′-AGG GAA GCA AGC ACT GGA TA; (Reverse) 5′-GCT GGC TGG AAT CAC ATC TT; *Dc-stamp* (Forward) 5′-GGG CAC CAG TAT TTT CCT GA; (Reverse) 5′-TGG CAG GAT CCA GTA AAA GG.

IMMUNOBLOTTING

Cell protein lysates were harvested from osteoclasts in modified RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% IGEPAL, 0.25% sodium deoxycholate, 1 mM EDTA) supplemented with Halt Protease &t

Phosphatase Inhibitor Cocktail (Thermo Scientific). Lysates were cleared by centrifugation at 12,000*g* at 4°C. Proteins were resolved by SDS-PAGE and transferred to PVDF membrane (Millipore). SMAD1/5/8 and SMAD4 antibodies were obtained from Cell Signaling Technology. HRP-conjugated anti-rabbit or anti-mouse were incubated with membranes, washed, and incubated with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare).

TRAP STAIN

Primary osteoclasts were fixed with 4 % paraformaldehyde (PFA) and washed with PBS. The cells were then stained for tartrate resistant acid phosphatase (TRAP) expression with tartrate 5 mg, Naphthol AS-MX phosphate, 0.5 mL M, M-Dimethyl formamide, 50 mL acetic acid buffer (1 mL acetic acid, 6.8 g sodium acetate trihydrate, 11.5 g sodium tartrate in 1 L water) and 25 mg Fast Violet LB salt. Cells were then observed and captured with light microscopy and the measurements were analyzed using NIH Image J.

QUANTITATING NUCLEI

TRAP stained multinuclear osteoclasts were stained with DAPI to visualize nuclei. Images of cells were captured with light microscopy and total nuclei were quantitated with NIH Image J. To calculate number of nuclei per cell, DAPI images were overlay with TRAP stained images to calculate number of nuclei per cell in TRAP positive cells containing three or more nuclei.

RESORPTION ASSAYS

Primary osteoclasts were plated on Corning Osteo Assay Surface plates at a density of 100,000 cells per well. Cells were allowed to fully differentiate. For resorption assays with BMP2 or dorsomorphin, osteoclasts were allowed to differentiate till day 4. Differentiation medium was supplemented with 50 ng/mL BMP2 (R&D Systems) or 1,200 nM dorsomorphin (Sigma) for 24 h. For all the resorption assays, the media was completely removed on day 5 and 100 μL/well of 10% bleach or TRAP stain was added and allowed to incubate at room temperature for 5 min. The bleach solution or TRAP solution was then aspirated and the wells were washed twice with 150 μL of dH_2O. The plate was then allowed to air dry completely at room temperature for 3–5 h. The wells were observed at 4× magnification for the formation of resorption pits and images were captured with light microscopy. Images were measured and analyzed using NIH Image J.

STATISTICS

All experiments were completed in triplicate and performed at least three times. The data shown are representative of the mean ± SD of all experiments. Student's *t*-test or 1 way ANOVA analysis followed by a Tukey's multiple comparison test were used to compare data; $P < 0.05$ indicates significance. Statistical analysis was performed using Prism 5 software for Mac OSX.

RESULTS

OSTEOCLAST DIFFERENTIATION AND ACTIVITY IS REDUCED IN SMAD4 AD-CRE CELLS

We previously demonstrated that treatment of osteoclasts with dorsomorphin inhibited their differentiation, which suggested that the canonical BMP/SMAD pathway is essential for osteoclast differentiation [Broege et al., 2013]. To further establish the importance of the canonical BMP signaling pathway during osteoclast differentiation, we isolated BMMs from *Smad4* floxed mice. Osteoclast precursors were infected with either a control adenovirus expressing EGFP, which will be referred to as Ad-Control, or an adenovirus expressing CRE recombinase, which will be referred to as Ad-CRE. Real time RT-PCR was used to measure *Smad4* gene expression following 48 h of infection by the adenoviruses (Fig. 1A). We detected a significant decrease in *Smad4* expression in Ad-CRE cells compared to Ad-Control infected osteoclasts ($P < 0.05$). Smad4 protein levels were analyzed by Western blot, which confirmed that osteoclasts infected with Ad-CRE expressed less Smad4 compared to Ad-Control cells (Fig. 1B).

To determine the functional significance of loss of Smad4 protein expression on osteoclast differentiation, BMMs were infected with either Ad-Control or Ad-CRE and differentiated in presence of M-CSF and RANKL for 5 days. Cultures were fixed, stained for TRAP (Fig. 1C), and quantified by counting (Fig. 1D) or measuring size (Fig. 1E) of the number of TRAP positive cells with three or more nuclei. Osteoclasts conditionally deleted for Smad4 expression (Ad-CRE) were half as numerous (Fig. 1D) and 2.5-fold smaller compared to Ad-Control cells (Fig. 1E).

Smad4 mediates both TGF-β and BMP signaling and both signaling pathways have been shown to play a role in cell apoptosis. To determine if the changes that we measured in number of TRAP positive multinuclear cells (Fig. 1D) were not due to increases in cell apoptosis, we measured total number of nuclei at day 5 of differentiation. We did not detect any significant differences in total number of nuclei in cells infected with control or CRE expressing virus (Fig. 1F). In addition, we measured the number of nuclei per cell and calculated that Ad-CRE cells had less nuclei per cell compared to Ad-Control cells (Fig. 1G). These data suggest that the lack of osteoclast differentiation in Smad4 Ad-CRE is not due to loss of cells but the inability of the cells to fuse into multinuclear cells.

To further determine the cause for decreased osteoclast differentiation that we observed in Fig. 1, we measured changes in osteoclast gene expression. We detected a 1.5-fold increase in *c-fos* expression in the Ad-Cre infected cells (Fig. 2A). Surprisingly, we found decreases in the other osteoclast markers in the Smad4 Ad-CRE cells that we measured. We quantified a 12-fold decrease in the expression of *Nfatc1* (Fig. 2B), a threefold decrease in *Dc-stamp* (Fig. 2C), and a fourfold decrease in *cathepsin K* (Fig. 2D) compared to the control infected cells. It has been shown by others that TGF-β signaling in osteoclasts affects NFATc1 expression but not by altering c-*fos* expression or NFATc1 localization [Fox et al., 2008]. Since Smad4 is a co-Smad for both BMP and TGF-β signaling, we wanted to determine if TGF-β signaling was affected in our Smad4 Ad-CRE cells. We analyzed phospho-Smad2/3 in Ad-Control and Ad-CRE Smad4 multinuclear osteoclasts. As shown in Fig. 2E, we detected less phospho-Smad 2 and Smad 3 by Western blot suggesting that TGF-β signaling is affected in Smad4 Ad-CRE osteoclasts.

We next asked if loss of Smad4 expression leads to changes in osteoclast activity. We cultured BMMs on calcium phosphate coated plates and measured pit number, average pit size and percent area resorbed. There were fewer resorption pits (Fig. 3A and B) and the

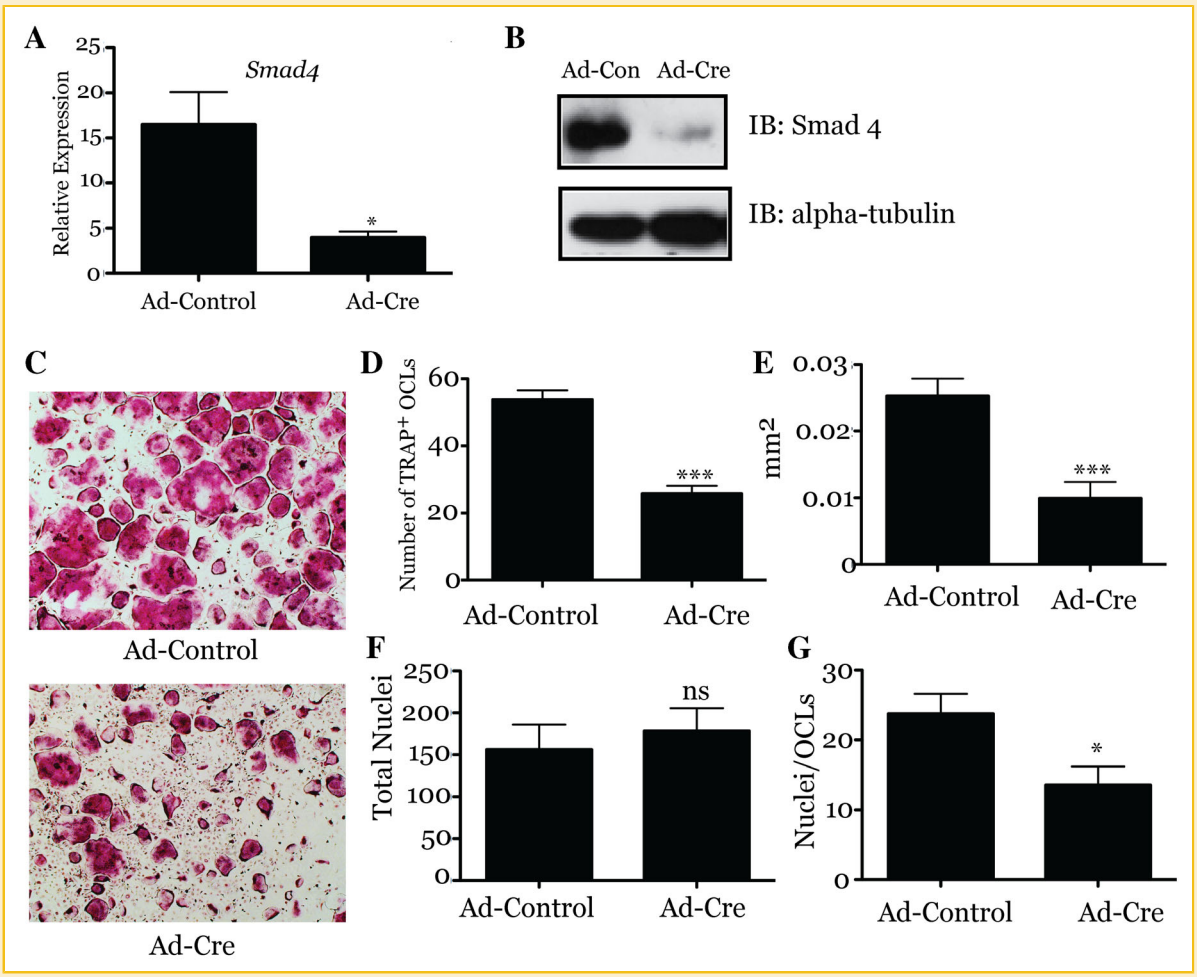

Fig. 1. Smad4 expression is required for osteoclast differentiation. BMMs were cultured from *Smad4*$^{fl/fl}$ mice and infected with either a control (Ad-Control) or CRE expressing adenovirus (Ad-CRE). A: Real time RT-PCR was used to measure *Smad4* gene expression following 48 h of infection by adenovirus. B: Smad4 protein levels were analyzed by Western blot. C–E: BMMs were differentiated in the presence of M-CSF and RANKL, TRAP stained, imaged and quantified. Only cells with three or more nuclei were quantified. F: Total nuclei quantitated by DAPI staining. G: Number of nuclei per TRAP positive cell containing three or more nuclei. Experiments were done at least three times and values represent the mean ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$.

average pit size was also reduced (Fig. 3C) when Smad4 was conditionally deleted (Ad-CRE) compared with the Ad-Control osteoclasts. The percent area resorbed for Smad4 Ad-Cre was decreased 28-fold compared to the Ad-Control cells ($P < 0.001$, Fig. 3D). We measured less multinuclear osteoclasts in our Ad-Cre infected cells; however, when percent area resorbed per number of osteoclasts was calculated, the resorption was still reduced 16-fold compared to Ad-Control cells (Fig. 3E).

OSTEOCLAST DIFFERENTIATION AND ACTIVITY IS REDUCED IN SMAD1/5 AD-CRE CELLS

Given that BMP-specific Smads1 and 5, but not 8, are expressed in osteoclasts (Fig. 4A), we hypothesized that these Smads would be critical for mediating the BMP response in osteoclasts. To test the necessity of Smad1 and 5 expression during osteoclast differentiation, we isolated BMMs from mice double homozygous for floxed alleles of *Smad1* and *Smad5*, which we refer to as Smad1/5 mice, and infected with either Ad-Control or Ad-CRE. Real time RT-PCR was used to measure *Smad1/5* gene expression 48 h after infection by adenovirus overexpressing the CRE recombinase (Ad-CRE). The results confirm a sevenfold decrease in *Smad1* expression (Fig. 4B) and 11-fold decrease in *Smad5* expression (Fig. 4B). Smad1/5/8 protein levels were analyzed by Western blot (Fig. 4C) and showed a decrease of Smad1/5/8 protein expression in Ad-CRE versus Ad-Control infected cells.

BMMs were differentiated in the presence of M-CSF and RANKL, then TRAP stained, imaged, and quantified. Osteoclasts conditionally deleted for Smad1/5 (Ad-CRE) were less numerous and smaller compared to Ad-Control cells (Fig. 4D–F). Similar to cells that were conditionally deleted for *Smad4*, total nuclei was similar in both Ad-control and Ad-CRE cells (Fig. 4G) as well as level of caspase 3/7 (data not shown); however, there was a significant decrease in the total number of nuclei per cell in the Ad-CRE Smad 1/5 osteoclasts (Fig. 4H).

To evaluate the cause of decreased osteoclast differentiation, we measured changes in expression of osteoclast-enriched genes. In the

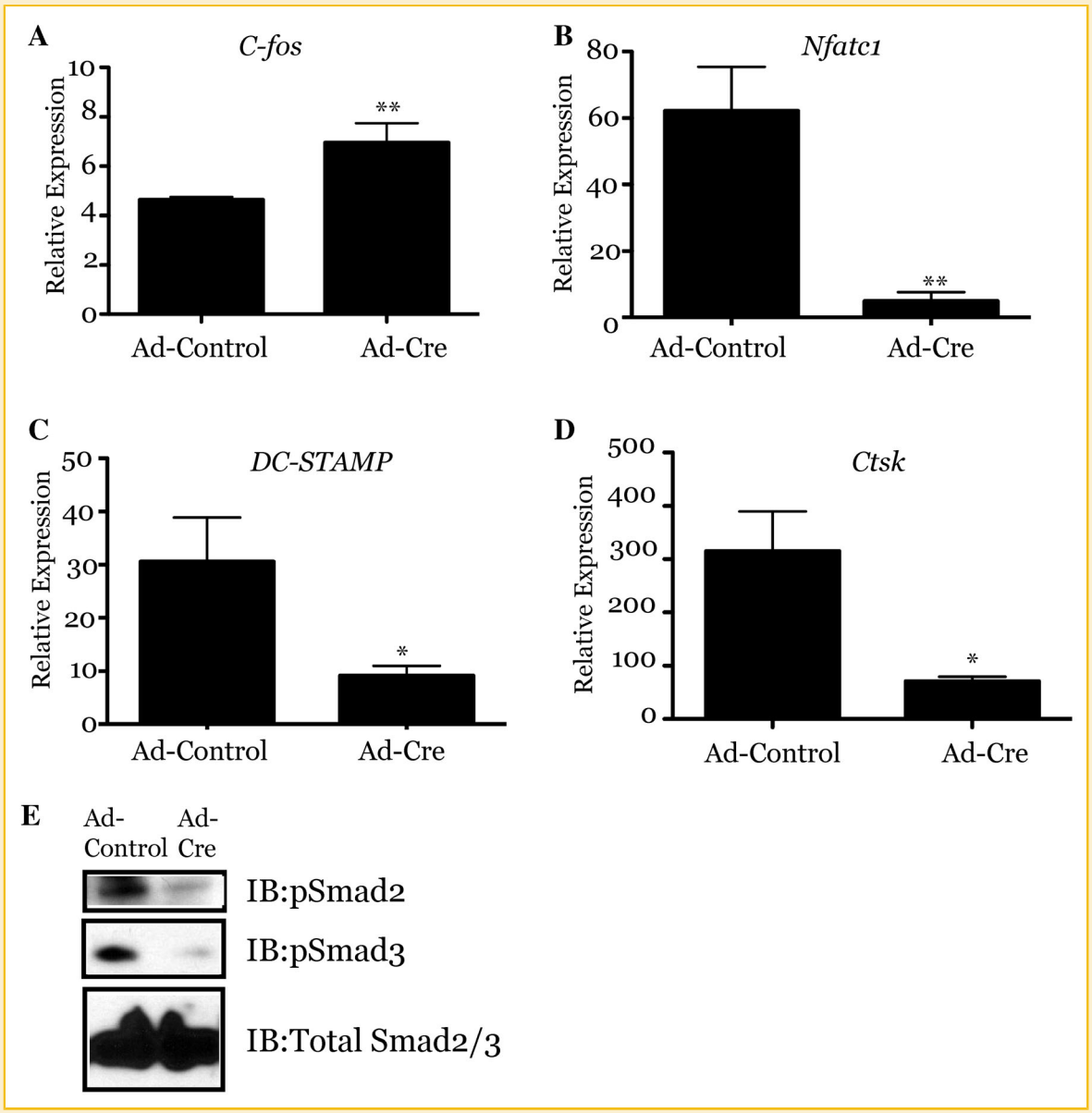

Fig. 2. Osteoclast gene expression is changed in Smad4 null osteoclasts. Real time RT-PCR was run on cDNA extracted from mature osteoclasts from *Smad4*$^{fl/fl}$ BMMs. A–D: *C-fos*, *Nfatc1*, *Dc-stamp* and *Ctsk* expression were measured as markers of osteoclast differentiation. E: Western blot of lysates from Ad-control and Ad-Cre Smad4 osteoclasts. Lysates were analyzed for phospho-Smad2, phospho-Smad3 and total Smad2/3 expression. Experiments were done at least three times and values represent the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.005$.

Smad1/5 Ad-CRE cells, both *c-fos* and *Nfatc1* expression were not significantly different between the control and CRE-infected cells (Fig. 5A and B); in contrast, expression of *Dc-stamp* and *cathepsin K* is reduced in Ad-CRE osteoclasts, 2-fold and 2.5-fold, respectively (Fig. 5C and D). To determine whether loss of Smad1/5 expression leads to changes in osteoclast activity, we cultured BMMs on calcium phosphate coated plates. There were fewer resorption pits and average size of the pits was reduced in the Smad1/5 Ad-CRE (Fig. 6A–C) compared to Ad-Control. The percent area resorbed for Smad1/5-Cre (Fig. 6D) was significantly decreased compared to control. As we measured for Smad4 cultures, percent area resorbed per number of osteoclasts was significantly reduced in Ad-Cre cultures (Fig. 6E). These results are consistent with what we observe in Smad4-null osteoclasts.

BMP2 STIMULATES OSTEOCLAST ACTIVITY

The Smad4 and Smad1/5 Ad-CRE osteoclasts were smaller and mostly mononuclear, and when we tested their ability to resorb calcium phosphate, we observed less area resorbed than with cells that were infected with control virus. This is not surprising since mononuclear osteoclasts have been shown to be capable of resorbing bone slices albeit less efficiently than multinuclear osteoclasts [Lee et al., 2006].

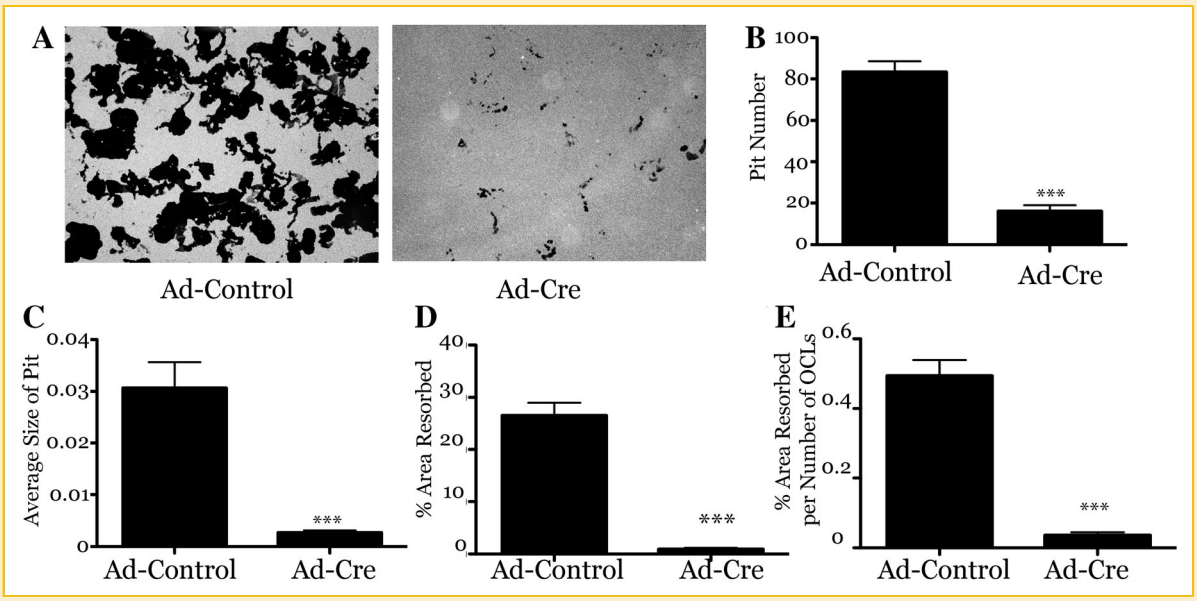

Fig. 3. Resorption is reduced in Smad4 null osteoclasts. BMMs were flushed from *Smad4*$^{fl/fl}$ mice, infected with control (Ad-Control) or CRE (Ad-CRE) expressing adenovirus and plated on calcium phosphate coated plates in the presence of M-CSF and RANKL. A: Representative images of calcium phosphate coated wells. Quantification of (B) number of pits, (C) average size of pits, (D) percent area resorbed (E) percent area resorbed per number of TRAP positive osteoclasts. Experiments were done at least three times and values represent the mean ± SD. *** $P < 0.001$.

To determine whether BMP interfered with the resorptive capacity of mature osteoclasts, we differentiated osteoclasts for 4 days and then treated these cultures with RANKL alone or RANKL + BMP2 (Fig. 7A) for 24 h. The time point of 24 h was chosen based on previous time course published by Kaneko et al. [2000] examining the effects of BMP2 on mature rabbit osteoclasts. BMP2 treatment stimulated the resorptive potential of mature osteoclasts, resulting in an increase in pit number (Fig. 7B), average size of the pits (Fig. 7C) and percent area resorbed (Fig. 7D). With the addition of BMP2 for 24 h, we detected no change in the number of mature osteoclasts when we TRAP stained the resorptive cultures and percent area resorbed per osteoclast number was still significantly increased with the addition of BMP2 (Fig. 7E).

To determine whether the canonical BMP pathway is responsible for the increase in resorption following BMP2 treatment, we treated mature osteoclasts with RANKL and DMSO vehicle, or RANKL and dorsomorphin, a chemical inhibitor that selectively blocks phosphorylation of Smad1/5/8 by the BMP receptor complex, and then tested their ability to resorb a calcium phosphate substrate. We chose to use the concentration of 1,200 nM because in our previous publication [Broege et al., 2013] we had shown that this concentration consistently blocked phosphorylation of pSMAD1/5/8. As shown in Fig. 7F–I, we detected a significant decrease in pit number (Fig. 7G), average size of pits (Fig. 7H) and percent area resorbed (Fig. 7I) when mature osteoclasts were treated with dorsomorphin. However, contrary to when osteoclasts were treated with BMP2, dorsomorphin treatment for 24 h decreased the number of TRAP positive osteoclasts. This therefore resulted in no significant change in percent area resorbed per number of osteoclast when comparing DMSO and dorsomorphin treated cultures (Fig. 7J).

DISCUSSION

In this study we show that expression of Smad4 and Smad1/5, proteins of the BMP canonical signaling pathway, are important for osteoclast differentiation. BMMs cultured from either *Smad4*$^{fl/fl}$ or *Smad1*$^{fl/fl}$/*5*$^{fl/fl}$ mice and infected with CRE-expressing adenovirus differentiated into TRAP positive mononuclear or smaller multinuclear cells compared to control infected cells. Both Smad4 and Smad1/5 enter the nucleus to regulate gene expression following BMP receptor activation. In osteoclasts that were conditionally deleted for *Smad4* or *Smad1/5*, we measured changes in expression of osteoclast differentiation markers such as *Dc-stamp* and *cathepsin K*; however, differences in gene expression between the two experimental systems was that while *c-fos* and *Nfatc1* expression was modestly increased and decreased, respectively in Smad4 Ad-CRE cells, *c-fos* and *Nfatc1* expression was unchanged in the Smad1/5 Ad-CRE osteoclasts. We found that osteoclasts conditionally deleted for Smad4 and Smad1/5 have less activity on a calcium phosphate substrate. We also demonstrated that BMP2 and dorsomorphin have the capacity to increase or decrease, respectively, the resorptive capacity of mature osteoclasts. Overall, our results strongly support the hypothesis that the canonical signaling arm of the BMP pathway plays an important role in osteoclast differentiation and function.

Understanding the mechanisms by which BMPs regulate osteoclast differentiation also has implications for human health. Recombinant BMPs are currently approved for use by the FDA in lumbar spinal fusions, long bone nonunion fractures, and sinus and alveolar ridge augmentation procedures. One of the major side effects of the clinical use of BMPs has been locally increased bone resorption and osteolysis leading to poor patient outcomes [Mroz et al., 2010].

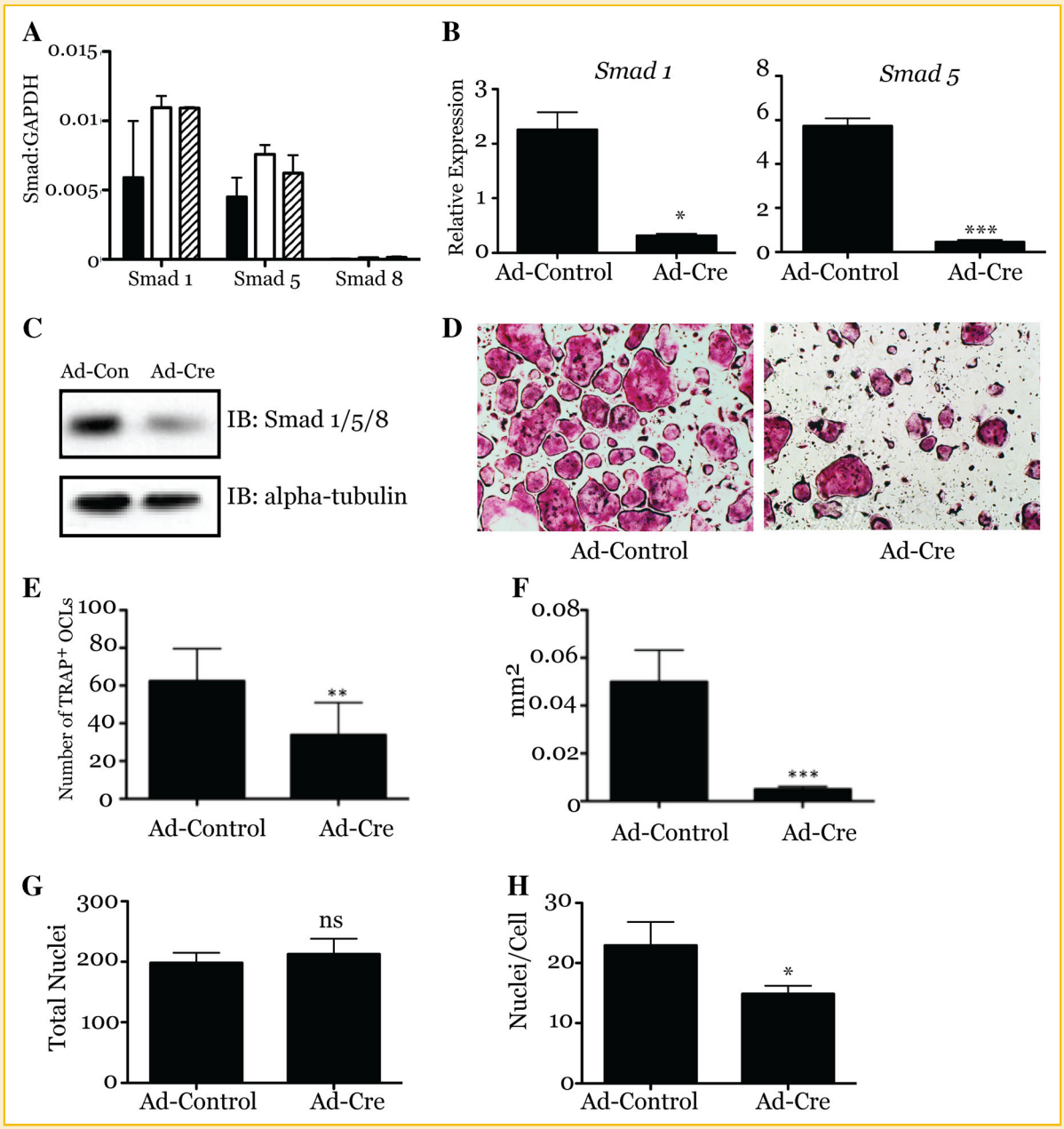

Fig. 4. Smad1/5 expression is required for osteoclast differentiation. A: Real time RT-PCR measuring expression of *Smad1*, *Smad5* and *Smad8* in osteoclasts at day 1 (black bar), day 3 (white bar) and day 5 (striped bar) following M-CSF and RANKL stimulation. BMMs were cultured from *Smad1/5*$^{fl/fl}$ mice and infected with either a control (Ad-Control) or CRE expressing adenovirus (Ad-CRE). B: Real time RT-PCR was used to measure *Smad1/5* gene expression following 48 h of infection by adenovirus. C: Smad1/5/8 protein levels were analyzed by Western blot. D–F: BMMs were differentiated in the presence of M-CSF and RANKL, TRAP stained, imaged and quantified. Only cells with three or more nuclei were quantified. G: Total nuclei quantitated by DAPI staining. H: Number of nuclei per cell in TRAP positive cells containing three or more nuclei. Experiments were performed at least three times and values represent the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.

In a previous publication we had demonstrated that addition of Noggin, a BMP antagonist could block RANKL induced osteoclast differentiation [Jensen et al., 2010]. This previous data along with our current study suggest that Smad signaling pathways are activated during osteoclast differentiation without the addition of exogenous BMP ligands. Previously we also demonstrated that osteoclasts secrete BMP2 and that expression of BMP2 mRNA increases as osteoclasts differentiate [Jensen et al., 2010]. Future studies in the lab will determine if other BMP ligands are expressed by osteoclasts and the significance of the expression of BMP ligands during osteoclast differentiation and activity.

We previously showed that osteoclasts with increased BMP signaling due to loss of TWSG1, a BMP inhibitor, are larger and more numerous compared to wild type osteoclasts [Rodriguez et al., 2009]. In that study we demonstrated that those larger osteoclasts had higher levels of phospho-Smad1/5/8 protein compared to wild type

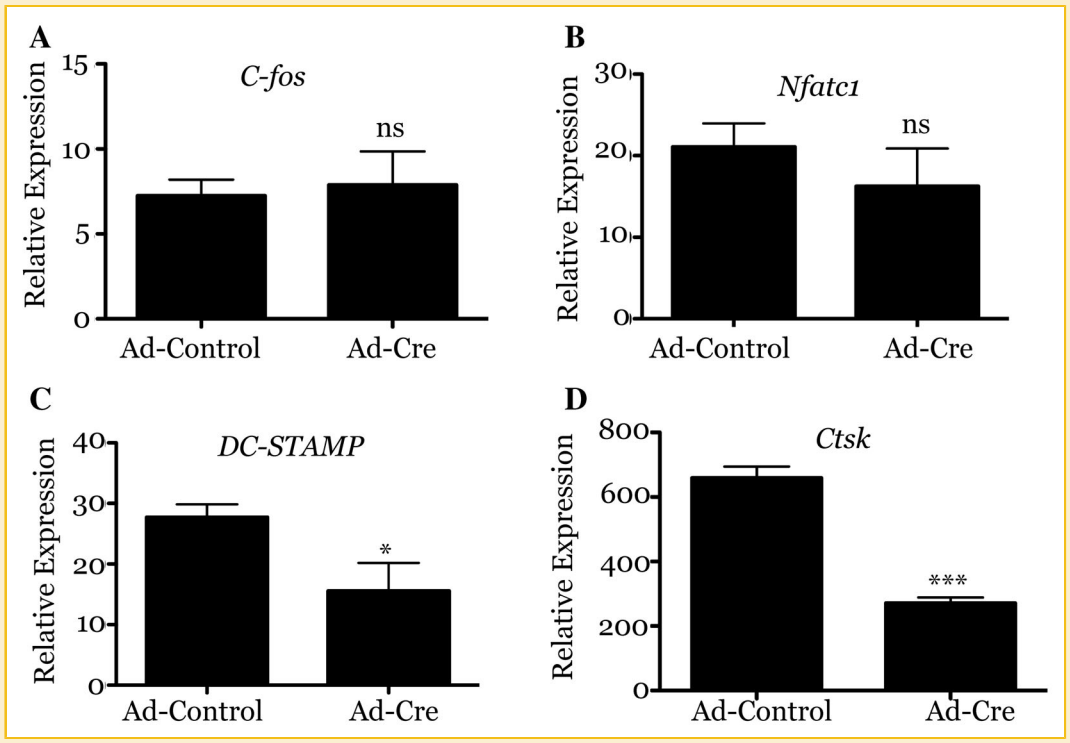

Fig. 5. Osteoclast gene expression is reduced in Smad1/5 null osteoclasts. Real time RT-PCR was run on cDNA extracted from mature osteoclasts from *Smad1/5*$^{fl/fl}$ BMMs. A–D: *C-fos*, *Nfatc1*, *Dc-stamp* and *Ctsk* expression were measured as markers of osteoclast differentiation. Experiments were performed at least three times and values represent the mean ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$.

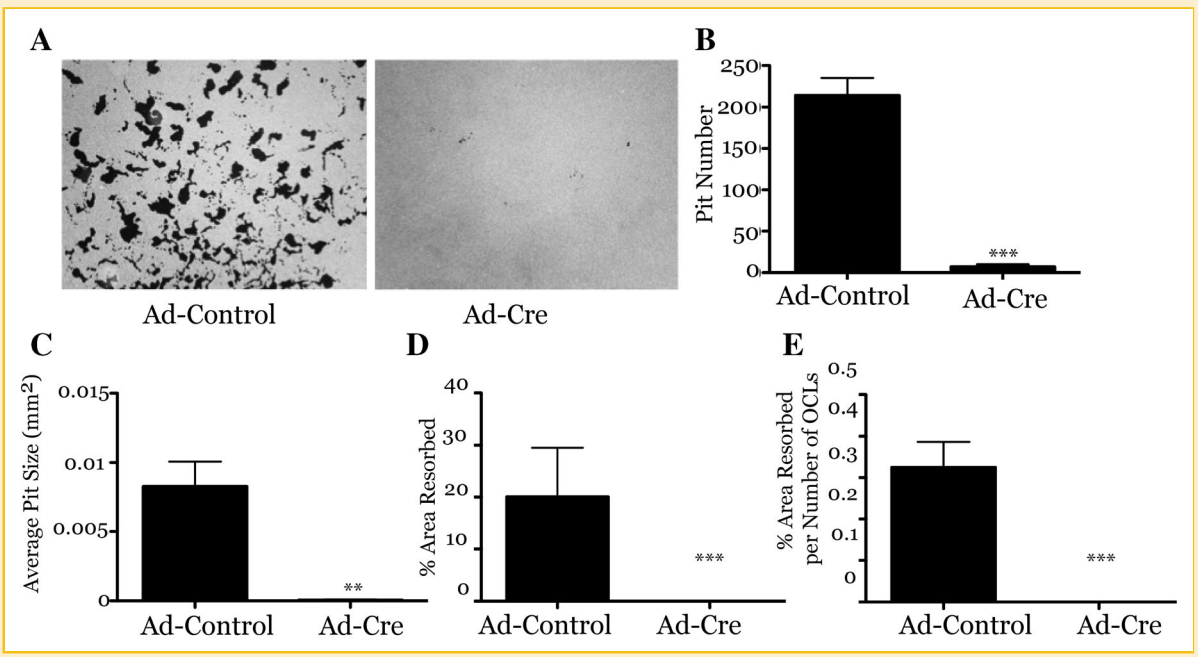

Fig. 6. Resorption is reduced in Smad1/5 null osteoclasts. BMMs were flushed from *Smad1/5*$^{fl/fl}$ mice, infected with control (Ad-Control) or CRE (Ad-CRE) expressing adenovirus and plated on calcium phosphate coated plates in the presence of M-CSF and RANKL. A: Representative images of calcium phosphate coated wells. Quantification of (B) number of pits, (C) average size of pits, (D) percent area resorbed (E) percent area resorbed per number of TRAP positive osteoclasts. Experiments were performed at least three times and values represent the mean ± SD. $^{**}P < 0.005$ and $^{***}P < 0.001$.

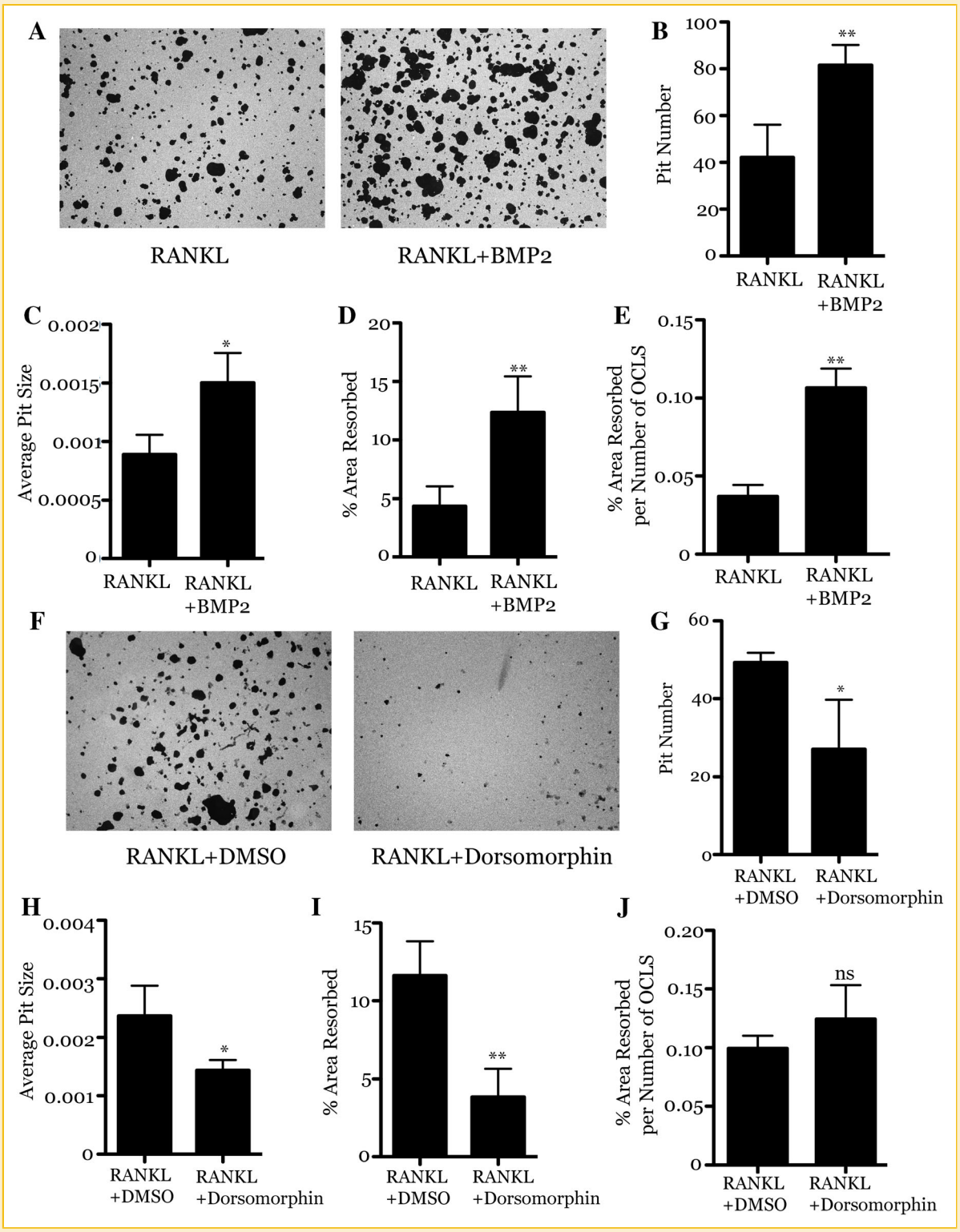

Fig. 7. Activity of mature osteoclasts is regulated by BMP signaling. BMMs were isolated from wild type mice and plated on calcium phosphate coated plated in the presence of M-CSF + RANKL until osteoclasts were multinuclear. A–E: Multinuclear osteoclasts were treated for 24 h with either M-CSF + RANKL (-BMP2) or M-CSF + RANKL + BMP2 (50 ng/mL) for 24 h. A: Representative images of calcium phosphate coated wells. B–E: Quantification of number of pits, average size of pits, percent area resorbed and percent area resorbed per number of TRAP positive osteoclasts. F–J: Multinucleated osteoclasts were treated for 24 h with either M-CSF + RANKL + DMSO or M-CSF + RANKL + Dorsomorphin (1,200 nM). F: Representative images of calcium phosphate coated wells. G–J: Quantification of number of pits, average size of pits, percent area resorbed and percent area resorbed per number of TRAP positive osteoclasts. Experiments were performed at least three times and values represent the mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.005$.

osteoclasts [Rodriguez et al., 2009]. Subsequently, in a follow up study we showed that phospho-Smad1/5/8 is initially detected in wild type osteoclast cultures around the time of fusion (day 3). Consistent with present work, we also showed that treatment of bone marrow macrophage cultures with dorsomorphin inhibited osteoclast formation [Broege et al., 2013]. This study extends our findings to demonstrate that the canonical signaling pathway, specifically Smad1 and 5, which are activated by BMPs, are necessary for osteoclast differentiation.

Studies are currently being conducted to evaluate the role of Smad 1/5 in osteoclastogenesis in vivo. Conditional knockout mice are being generated using *LysM* and *c-fms* cre. Previous studies determining the role of Smad1/5 in limb development demonstrated that there is a positive feedback loop between canonical BMP signaling and BMP receptors [Retting et al., 2009]. The authors noted that Smad4 expression was not altered in the Smad1/5 double knockouts; however, the expression of type I and type II BMP receptors was reduced. The changes that we observe in our Smad1/5 knockdown osteoclasts may be a consequence of reduction in BMP receptor expression.

Smad4 is a co-Smad that is involved in both BMP and TGF-β signaling. TGF-β is considered an important regulator of osteoclasts and osteoblasts. It has been shown that TGF-β is necessary to commit monocytes to the osteoclast lineage [Karsdal et al., 2003]. Similar to our studies with BMP, TGF-β augments RANKL induced osteoclast formation [Fuller et al., 2000]. Due to Smad4′s role in both the BMP and the TGF-β pathways, we cannot discount that the changes that we measure in osteoclast differentiation in Smad4 conditionally deleted osteoclasts are due to changes in the response of the osteoclast precursors to TGF-β. In fact, we demonstrated that Ad-CRE Smad4 osteoclasts have less detectable expression of phospho-Smad 2 and Smad 3 (Fig. 2E). Changes in phospho-Smad 2/3 expression were not detected in Smad1/5 Ad-CRE osteoclasts (data not shown). The changes in phospho-Smad 2/3 expression may be due to the inability of Smad4 to form a heterocomplex with either Smad 2 or Smad 3, which has been shown to lead to instability of Smad 2/3 protein [Shi et al., 1997; Xu and Attisano, 2000].

For most parameters assessed in our current study, Smad4- and Smad1/5-deficient cells behaved similarly; however, differences of note were expression of *c-fos* and *Nfatc1*, which were only affected in Smad4 conditionally deleted cells. Surprisingly we measured a 1.5-fold increase in *c-fos* expression but a 12.5-fold decrease in *Nfatc1* expression by RT-PCR in Smad4 Ad-Cre osteoclasts (Fig. 2). Fox et al. [2008] demonstrated that osteoclast cultures treated with M-CSF, RANKL and TGF-β had an increase in NFATc1 protein expression compared to osteoclasts treated with only M-CSF and RANKL; however, there was no change in c-fos protein expression suggesting TGF-β/Smad4 regulates NFATc1 protein expression independent of c-*fos* expression. Our data similar to the Fox et al. study suggests that *Nfatc1* expression is regulated by TGF-β/Smad4 independent of changes in *c-fos* expression. However, experiments are ongoing in the lab to try and determine the mechanism(s) by which TGF-β/Smad4 regulates *Nfatc1* expression.

Additionally, our real time RT-PCR data (compare Figs. 2 and 5) suggest that *Nfatc1* expression is more greatly influenced by TGF-β signaling; whereas, BMP signaling through Smad1/5 mediates its effects through regulating genes involved in fusion such as *Dc-stamp*. This conclusion is supported by our recent study where we treated osteoclasts with dorsomorphin and saw a reduction in *Dc-stamp* expression but no change in *Nfatc1* [Broege et al., 2013]. However, we will need to do further analysis with ChIP to determine if Smads are directly or indirectly regulating osteoclast gene expression.

In a study with rabbit osteoclasts, Kaneko et al. [2000] demonstrated that both BMP2 and BMP4 increased bone resorption, but it remained unexplored whether canonical signaling components were required for this effect. Fong et al. [2013] recently demonstrated that BMP9 added to mature osteoclasts enhanced their ability to resorb bovine bone slices but did not affect formation. The authors further demonstrated that BMP9 inhibits osteoclast apoptosis, which may explain the stimulatory effect on bone resorption [Fong et al., 2013]. Similarly, we found that BMP2 also stimulates resorption in mature osteoclast cultures (Fig. 7). Upon further analysis, our studies with dorsomorphin and mature osteoclasts suggest that canonical BMP signaling may play a role in BMP2 enhancement of osteoclast activity by preventing apoptosis of mature osteoclasts (Fig. 7J). Collectively our data suggest that the canonical pathway may not only play a role in osteoclast precursor fusion, but also in regulating osteoclast activity.

CONCLUSIONS

From this study we conclude that expression of Smad1/5 and Smad4 is essential for osteoclast differentiation and activity. BMP2 can enhance osteoclast activity and that the enhancement is partially through the canonical BMP signaling pathway.

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