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Antagonism between Bone Morphogenic Protein and Activin signaling pathways in osteoprogenitor cells

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Abstract

Osteoporosis is a disease characterized by low bone mineral density due to the rate of bone resorption exceeding that of bone formation. Substantial evidence indicates the Bone Morphogenetic Protein (BMP) pathway promotes bone formation through action of the effectors SMAD1/5/8 while the Activin pathway negatively influences bone mass through action of the effectors SMAD2/3. Recent studies suggest that BMPs and Activins regulate bone mass in a see-saw-like mechanism. Here, we seek to test this hypothesis *in vitro* via signaling responsiveness assays using pathway-specific western blot analyses in the osteogenic murine bone marrow stromal cell line W-20-17. We first confirmed that W-20-17 cells exhibit basal activation of SMAD1/5/8 and SMAD2/3 under serum-restricted conditions. Moreover, treatment with Follistatin, which sequesters Activin ligands in the extracellular environment, leads to an increase in BMP pathway activation. To determine the molecular mechanism allowing for this relationship, we treated W-20-17 cells with SB431542, which is an intracellular inhibitor of Activin signaling that functions downstream of receptor engagement, and found no effect on BMP pathway activation. In contrast, treatment of W-20-17 cells with BMP pathway inhibitor Noggin had no effect on Activin pathway activation despite robust inhibition of BMP signaling. Collectively, our results suggest Activin-mediated repression of BMP signaling in these cells is ligand-dependent but occurs upstream of SMAD2/3 activation. Gene expression analyses indicate that W-20-17 cells express Activin A and its receptors ALK4, ACVR2A, and ACVR2B. Given that ACVR2A and ACVR2B also have high affinity for BMP ligands, this raises the possibility that Activin-mediated repression of BMP signaling may occur via competition for a shared pool of receptors. Over-expression studies coupled with osteoblast activity assays are currently underway to examine this hypothesis. Collectively, our work seeks to elucidate the mechanism(s) that regulate antagonism of BMP and Activin signaling pathways in the osteoblast lineage to identify novel opportunities for treating low bone mass in humans.

BMP & Activin Signaling Pathways

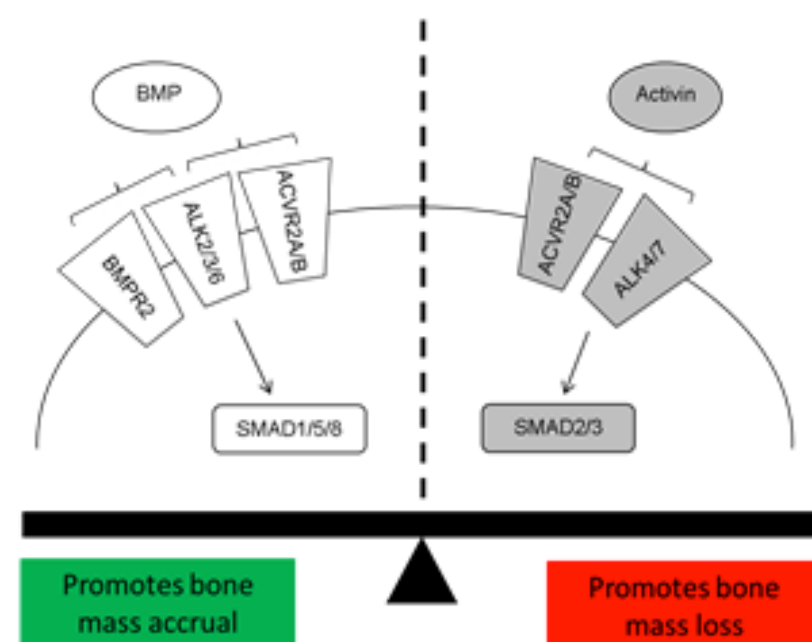

Figure 1. The BMP and Activin signaling pathways are initiated by ligand engagement with hetero-complexes of type 1 and type 2 receptors. In the BMP pathway, ALKs2, 3, and 6 are type 1 receptors, each of which activate the downstream effectors SMADs1, 5, and 8 in response to activation by a type 2 receptor (such as BMPR2). Similarly, Activin ligands induce the type 1 receptors ALK4 or ALK7 to activate SMADs2 and 3. Notably, ACVR2A and ACVR2B may serve as type 2 receptors for either. We conceptualize the effects of these pathways in the skeleton as a see-saw mechanism, with BMPs generally promoting bone mass accrual and Activins generally promoting bone mass loss.

BMP inhibition upstream of receptor engagement

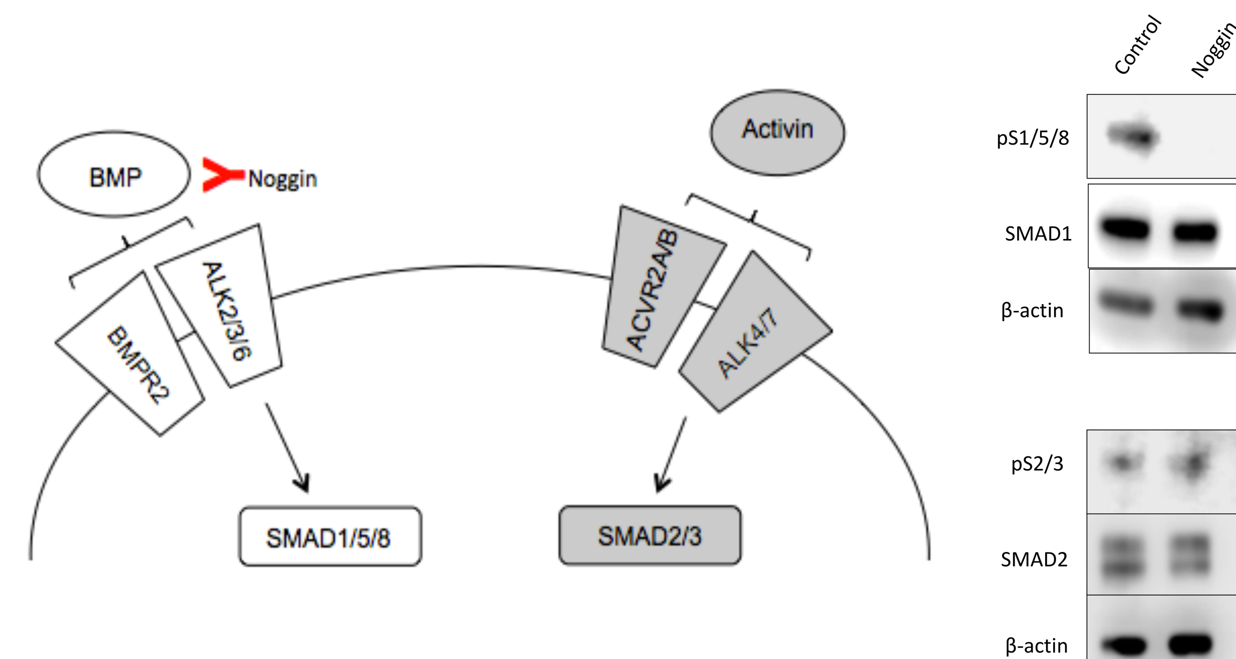

Figure 2. W-20-17 murine bone marrow stromal cells were serum-restricted for twenty-four hours then treated with 250 ng/ml Noggin for four hours. Western blots were then performed for BMP pathway activation level (phosphorylated SMAD1/5/8 compared to total SMAD1), and Activin pathway activation level (phosphorylated SMAD2/3 compared to total SMAD2) with beta-actin acting as a control. These results indicate that Noggin treatment had no effect on Activin pathway activation despite robust inhibition of BMP signaling. n=3 per group.

Activin inhibition upstream of receptor engagement

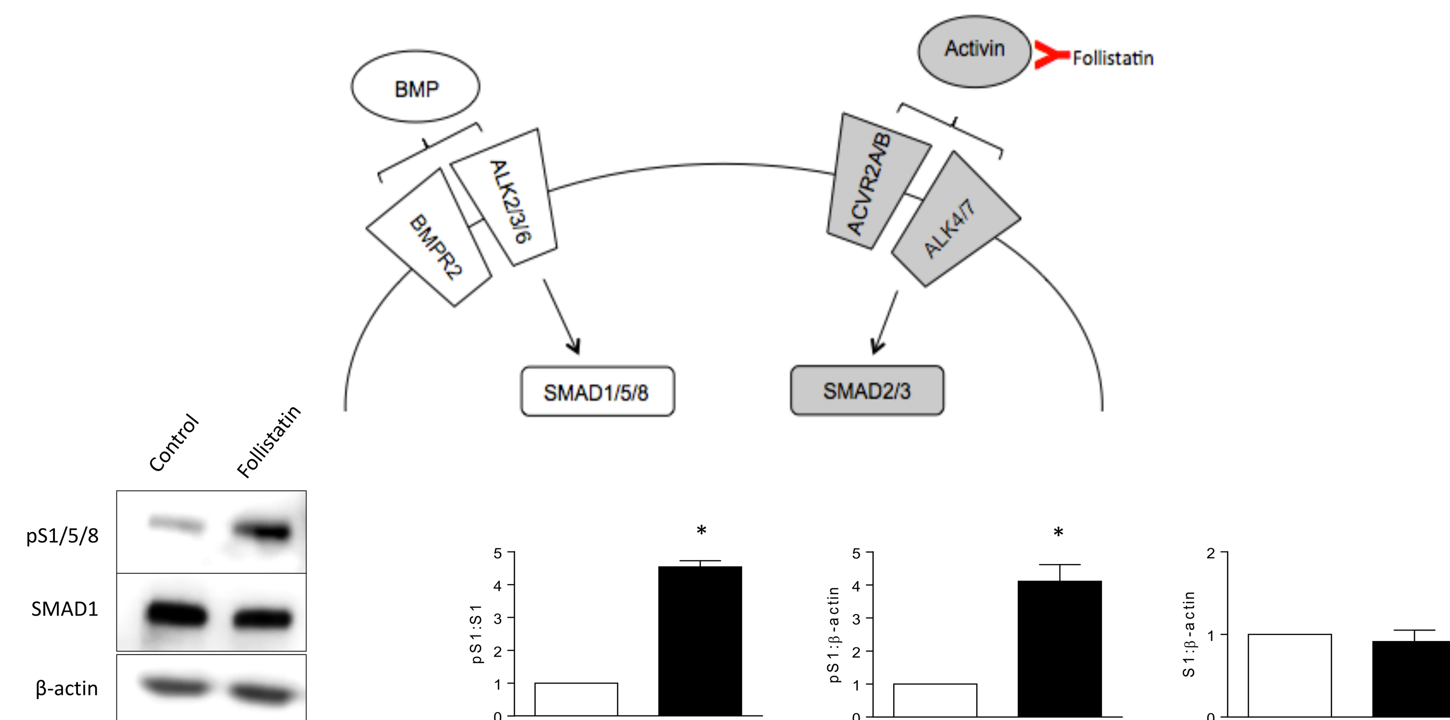

Figure 3. W-20-17 cells were serum-restricted for twenty-four hours then treated with 250 ng/ml Follistatin for four hours. Western blots were then performed for BMP pathway activation level (phosphorylated SMAD1/5/8 compared to total SMAD1), with beta-actin acting as a control. These results indicate that Follistatin treatment alleviates Activin-mediated repression of BMP signaling. * indicates p<0.05; n=3 per group.

Activin inhibition downstream of receptor engagement

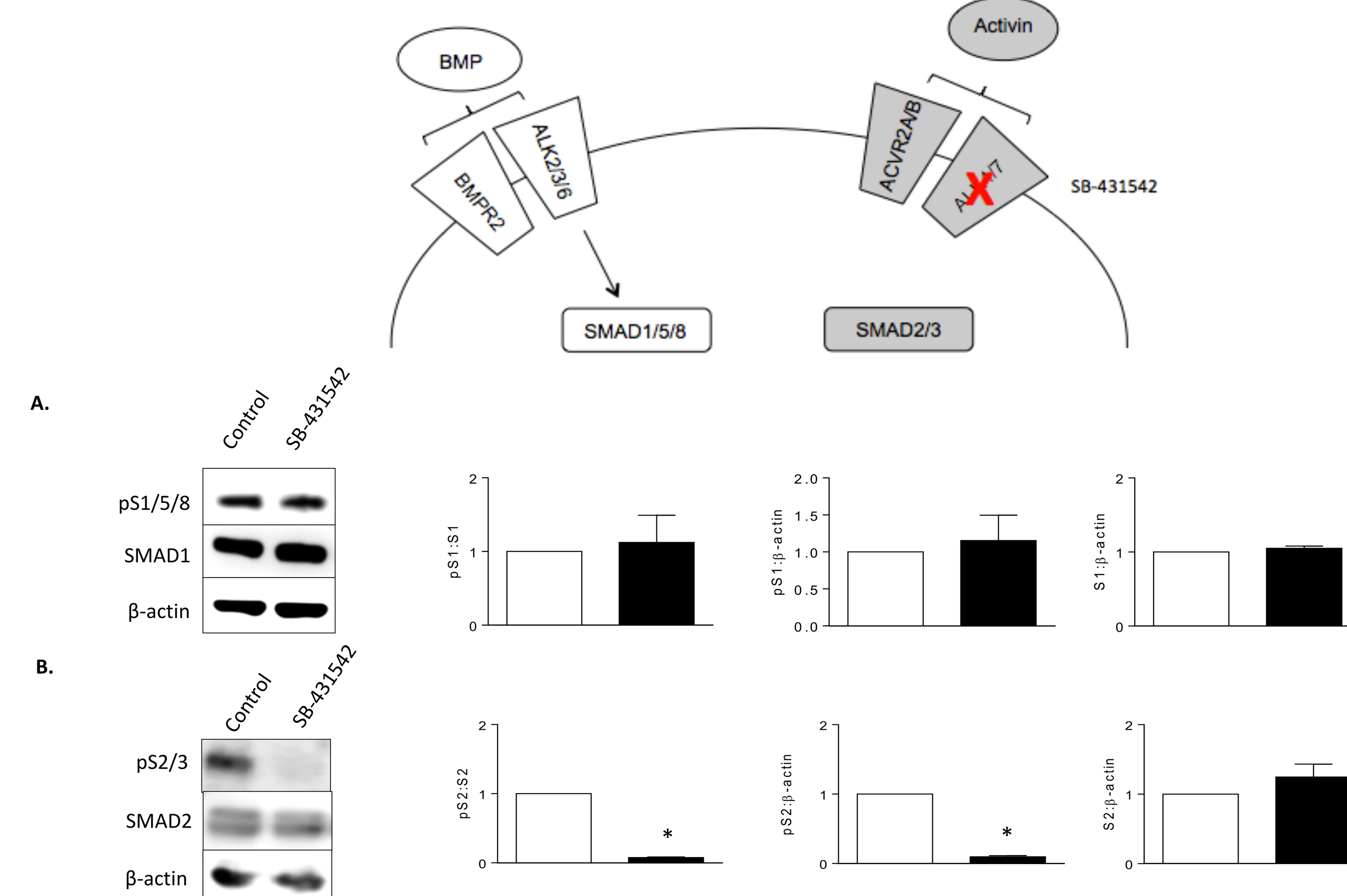

Figure 4. W-20-17 cells were serum-restricted for twenty-four hours then treated with 10 uM SB431542 for four hours. Western blots were then performed for BMP pathway activation level (**A**, phosphorylated SMAD1/5/8 compared to total SMAD1) or Activin pathway activation (**B**, phosphorylated SMAD2/3 compared to total SMAD2). These results indicate that SB431542 treatment does not alleviate Activin-mediated repression of BMP signaling. * indicates p<0.05; n=3 per group.

Working Interpretations

ACVR2A/B modulates signals for the TGF-beta superfamily of ligands, including BMP and Activin. Activin has been shown to counteract BMPs that signal through the ACVR2A/B receptors, however, Activin has not been shown to counteract BMPs that signal through BMPR2.

Noggin is a BMP-specific antagonist protein, which upon addition to the W-20-17 cells resulted in a decrease in phosphorylation of SMAD 1/5/8 and no effect on the phosphorylation of SMAD 2/3. Here we observed that BMP inhibition upstream of the receptor does not impact SMAD 2/3 phosphorylation. Such also supports the signaling competency of W-20-17 cells at the basal level, as seen in the control group.

Follistatin (FST) is an Activin binding protein, which upon addition to the W-20-17 cells resulted in an increase in the phosphorylation of SMAD 1/5/8. Here we observed that Activin inhibition upstream of the receptor allows for the upregulation of BMP signaling. SB-431542 is an intracellular inhibitor of Activin signaling, which upon addition to the W-20-17 cells resulted in a loss of SMAD 2/3 phosphorylation.

Collectively, our data suggest that Activin mediated repression of BMP signaling is *ligand dependent but occurs upstream of effector activation.*

Current Work 1: Transduction/Overexpression

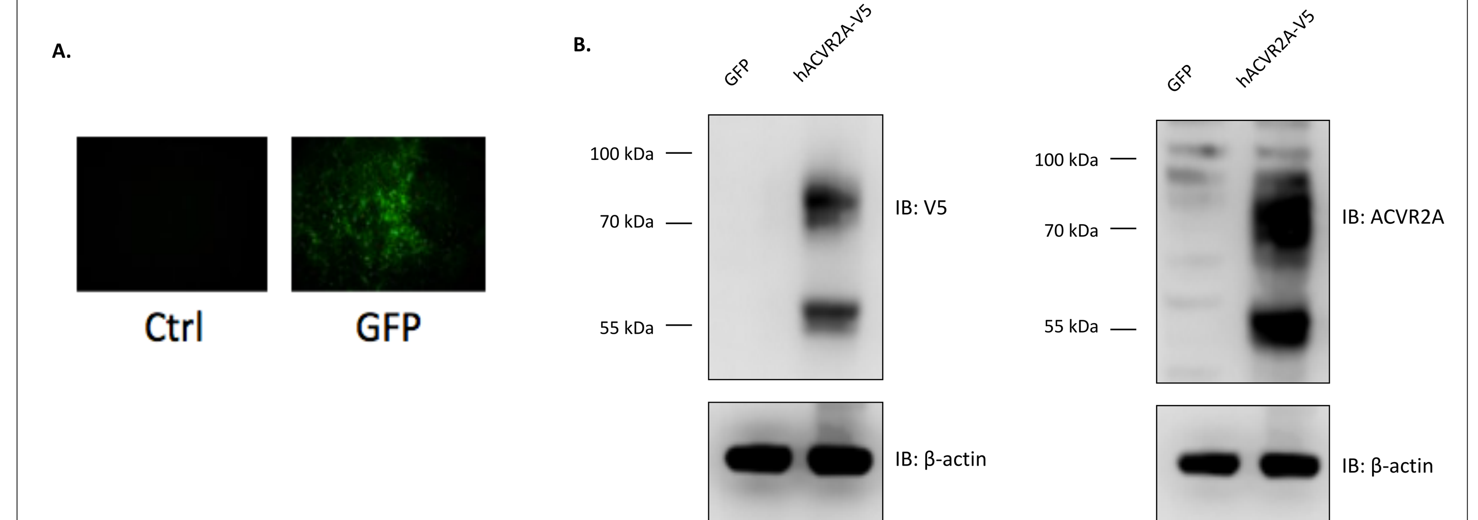

Figure 5. A: Optimization of lentiviral transduction method for W-20-17 cells. Fluorescent micrographs from W-20-17 cells transduced with GFP lentivirus (right) compared to non-transduced control (Ctrl). **B:** W-20-17 cells were treated with a lentivirus carrying cDNA encoding C-terminal V5-tagged human ACVR2A (ACVR2A-V5) or GFP (as a negative control). Cells were selected using puromycin and overexpression confirmed by western blot analysis. Western blots (V5, left; ACVR2A, right) are shown confirming expression of a V5-tagged gene for hACVR2A in transduced W-20-17 cells compared to the beta-actin control.

Current Work 2: Inhibition of specific Activin ligands

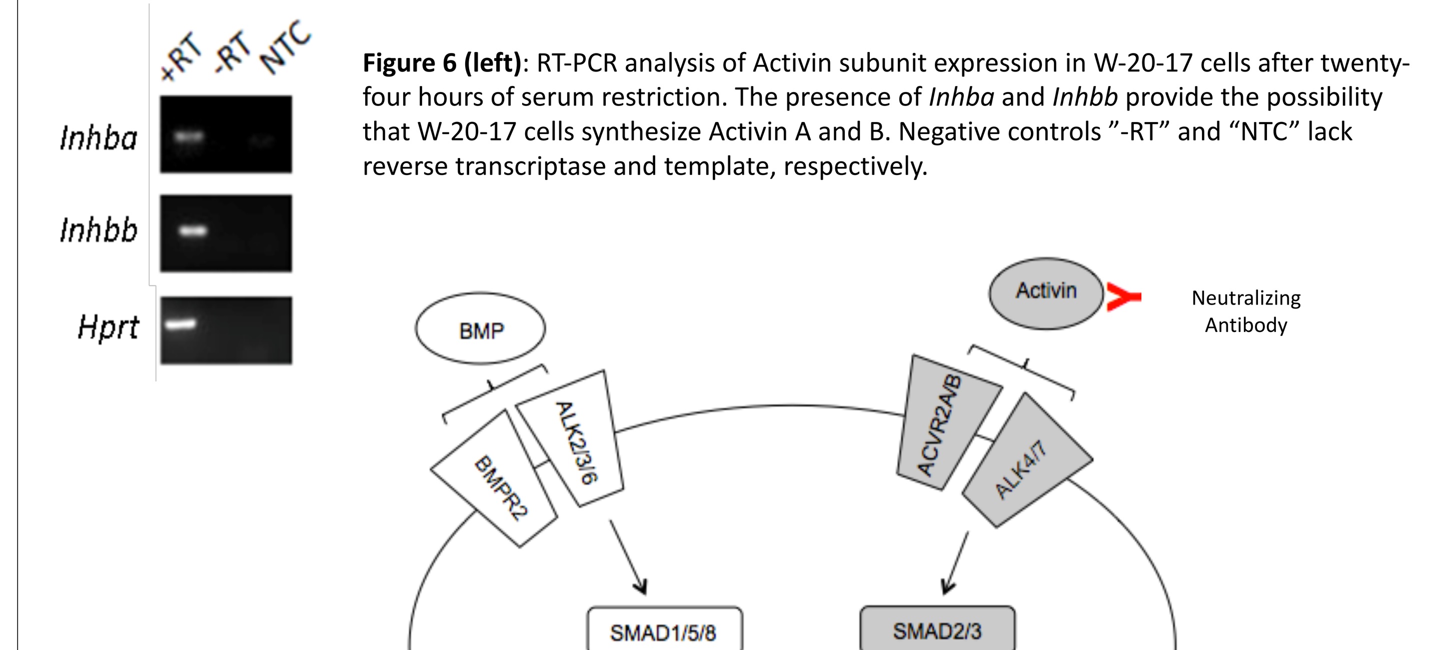

Figure 6 (left): RT-PCR analysis of Activin subunit expression in W-20-17 cells after twenty-four hours of serum restriction. The presence of *Inhba* and *Inhbb* provide the possibility that W-20-17 cells synthesize Activin A and B. Negative controls "-RT" and "NTC" lack reverse transcriptase and template, respectively.

Figure 7. W-20-17 cells will be serum-restricted for twenty-four hours and then treated with neutralizing antibodies against Activin A and Activin B for four hours. The cells will be lysed and western blot analyses performed to examine the potential effect on BMP pathway activation, and results will be compared against treatment with Follistatin. We hypothesize that, similar to Follistatin, treatment of W-20-17 cells with anti-Activin ligand antibodies will alleviate Activin-mediated repression of BMP signaling and we will see an increase in the phosphorylation of SMAD1/5/8.

Current Work 3: Follistatin treatment in cells overexpressing ACVR2A

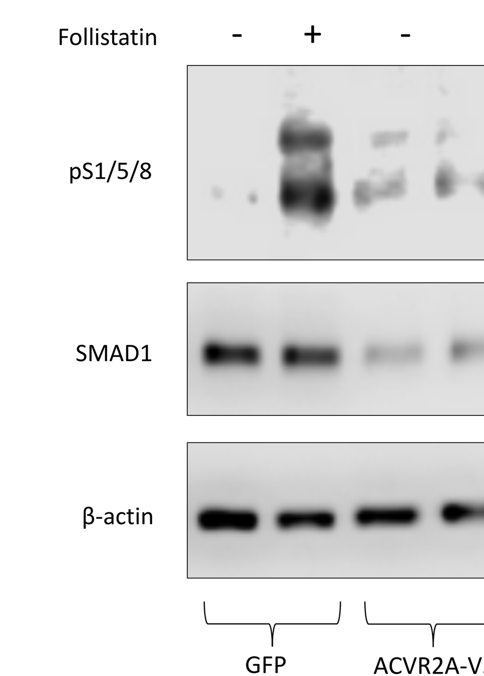

Figure 8. W-20-17-GFP+ and W-20-17-ACVR2A-V5+ cells were serum-restricted for twenty-four hours and then treated with 250 ng/ml Follistatin for four hours to examine the potential effect of ACVR2A overexpression on BMP pathway activation. Western blot analyses were performed for BMP pathway activation level (phosphorylated SMAD1/5/8 compared to total SMAD1) with beta-actin acting as a control. These preliminary results indicate that Follistatin treatment has no effect on cells transduced with lentivirus overexpressing ACVR2A. We anticipate that these results provide evidence that Activin-mediated repression of BMP signaling occurs at the level of receptor competition.

Acknowledgements

For a video presentation of this poster and to join the conversation:
http://bit.ly/2nPBTHS

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