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Activin Type 2 Receptor Restoration in MSI-H Colon Cancer Suppresses Growth and Enhances Migration With Activin

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Abstract

Background & Aims—Colon cancers with high-frequency microsatellite instability (MSI-H) develop frameshift mutations in tumor suppressors as part of their pathogenesis. *ACVR2* is mutated at its exon 10 polyadenine tract in >80% of MSI-H colon cancers, coinciding with loss of protein. *ACVR2* transmits the growth effects of activin via phosphorylation of SMAD proteins to affect gene transcription. The functional effect of activin in colon cancers has not been studied. We developed and characterized a cell model in which we studied how activin signaling affects growth.

Methods—*hMLH1* and *ACVR2* mutant HCT116 cells were previously stably transferred with chromosome 2 (HCT116+chr2), restoring a single regulated copy of wild-type *ACVR2* but not *hMLH1*. Both HCT116+chr2 and parental HCT116 cells (as well as HEC59 and *ACVR2* and *hMSH2* complemented HEC59+chr2 cells) were assessed for genetic complementation and biologic function.

Results—HCT116+chr2 cells and HEC59+chr2 cells, but not *ACVR2*-mutant HCT116 or HEC59 cells, acquired wild-type *ACVR2* as well as expression of *ACVR2* wild-type messenger RNA. Complemented *ACVR2* protein complexed with *ACVR1* with activin treatment, generating nuclear phosphoSMAD2 and activin-specific gene transcription. *ACVR2*-restored cells showed decreased growth and reduced S phase but increased cellular migration following activin treatment. *ACVR2* small interfering RNA reversed these effects in complemented cells.

Conclusions—*ACVR2*-complemented MSI-H colon cancers restore activin-SMAD signaling, decrease growth, and slow their cell cycle following ligand stimulation but show increased cellular migration. Activin is growth suppressive and enhances migration similar to transforming growth

factor β in colon cancer, indicating that abrogation of the effects of activin contribute to the pathogenesis of MSI-H colon cancers.

Approximately 15% of colon cancers display microsatellite instability and have a unique genetic makeup consisting of frameshifted target genes that drive their pathogenesis. Key growth and apoptosis regulators, such as TGFBR2 and BAX, contain coding microsatellites that become mutated with subsequent loss of transforming growth factor (TGF)- β signaling or apoptotic responses, respectively.¹ More recently, *ACVR2* was shown to be mutated in the majority of high-frequency microsatellite instability (MSI-H) colon cancers, with loss of protein expression.² However, the functional consequences of loss of activin signaling in MSI-H colon cancer are unknown.

Activin is a member of the TGF- β superfamily that can regulate cell differentiation, proliferation, and apoptosis in a variety of cancer cell types.^{3,4} Similar to TGF- β , activin utilizes 2 types of cell surface receptors to transduce its signal. Both activin receptor 1 (*ACVR1*) and activin receptor 2 (*ACVR2*), as well as *ACVR2B*, are trans-membrane proteins with ligand-binding activity in the extracellular domain and serine/threonine kinases in their cytoplasmic domain. *ACVR2* is located on chromosome 2 and harbors 2 coding polyadenine tracts. *ACVR2B*, located on chromosome 3, is 69% identical to *ACVR2* and has similar binding characteristics to *ACVR2* but does not contain any polyadenine tract. Control of expression of both receptors appears to occur in a tissue- and gene-specific manner during human development.⁵ Potential ligands for *ACVR2* include activin A, activin B, and inhibin A, but not TGF- β 1.⁶ Activin A is secreted by most cell types, is commercially available, and is the activin ligand most commonly used for studies published in the literature.

Activin signaling induces growth inhibition in T47D human breast cancer cells^{3,7} and LNCaP prostate cancer cells⁴ and induces apoptosis in hematopoietic and myeloid leukemia cells.^{8,9} Cell adhesion proteins are up-regulated through activin signaling in chicken limb buds, suggesting a role in cell anchoring.¹⁰ In normal keratinocytes, activin induces expression of *Mad1*, a c-MYC proto-oncogene antagonist.¹¹ While *Mad1* overexpression inhibits the activity of activin and TGF- β -induced transcriptional stimulation in a variety of mammalian cells, overexpression of *Mad3* potentiates activin-induced transcription.¹² Additionally, activin signaling may cause direct down-regulation of the proto-oncogene *c-MYC* in prostate cancer cells⁴ as well as an increase in hypophosphorylated retinoblastoma protein in hepatoma cell lines, indicative of decreased S-phase entry.¹³ In the same model, p21 induction was observed following activin treatment. Defective activin signaling drives carcinogenesis, and blocking activin signaling in breast cancer cells abrogates activin-mediated growth arrest.³ Activin has been implicated in tumor formation of the pituitary and ovaries,^{14,15} and loss-of-function mutations in *ACVR1* have been described in human pituitary tumors.¹⁶ Somatic mutations in *ACVR1* have been demonstrated in pancreatic cancer,¹⁷ and somatic mutation and loss of heterozygosity at the *ACVR2* locus have been described in pancreatic carcinoma.¹⁸ Mutated *ACVR2* abolishes activin-mediated erythroid differentiation,¹⁹ further underscoring its role of a potential tumor suppressor.

Mutant *ACVR2* has been implicated in colon carcinogenesis. Through a genome-wide search, 58% of 46 colon cancer cell lines with MSI-H were found to harbor mutant *ACVR2*.²⁰ A second study revealed that 92% of 24 colon cancer cell lines and xenografts with MSI-H were *ACVR2* mutated,¹⁸ all primarily due to a frameshift in the A₈ tract of exon 10. We demonstrated a similarly high mutational rate in primary colon cancer specimens, accompanied by loss of *ACVR2* protein expression in the majority of colon cancers.² Our primary human tumor data underscore the potential importance of activin signaling in colon cancer. To date, little is known about the mechanisms and cellular effects of activin signaling in colon cancer. Here, we explored the effects of *ACVR2* restoration in cell models of MSI-H in which *ACVR2* is mutated. We found that activin is growth suppressive in our single-copy, gene-complemented colon cancer cell models and enhances cellular migration. These findings are similar to the effects of TGF- β in colon cancer cells and indicate that loss of activin signaling contributes to the pathogenesis of MSI-H colon cancers.

Materials and Methods

Ligand

Activin A (R&D, Minneapolis, MN) was reconstituted in phosphate-buffered saline (PBS) and used at a final concentration of 25 ng/mL as described elsewhere.²¹

Antibodies

Rabbit anti-TyrGly m*ACVR2* (482–494) (a generous gift from W. Vale, Salk Institute), with its target epitope in the C-terminus region of *ACVR2* (beyond the resulting truncation from frameshift in exon 10), was used for Western blotting at 1:800; rabbit anti-TyrGly *ACVR1* (474–494) and rabbit anti-TyrGly *ACVR2B* (524–536) (also gifts from W. Vale, Salk Institute) were both used at 1:800; p*SMAD2* (Cell Signaling, Beverly, MA) was used at 1:250; *SMAD2* (Upstate, Lake Placid, NY) was used at 1:400; and α -tubulin and α -histone (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:250 and 1:500, respectively

Cell Lines

Both *ACVR2*-mutated HCT116 cells and *ACVR2*-restored HCT116+chr2 cells were created as described previously.²² We also utilized another cell line, the MSI-H endometrial cell line HEC59, as well as its microsatellite stable and *ACVR2*-restored counterpart HEC59+chr2,²³ the TGF- β -responsive human colon carcinoma cell line FET,²⁴ and the *ACVR2*- and *ACVR1*-expressing pancreatic cancer CAPAN-1 cells.²⁵ While the results shown are all from one clone of the respective cell lines, experiments were repeated using multiple clones.

Tissue Culture

HCT116 colon cancer and CAPAN-1 pancreatic cancer cells were maintained in Iscove's modified Dulbecco's medium (Invitrogen Corp, Carlsbad, CA) with 10% fetal bovine serum and penicillin G (100 U/mL)/streptomycin (100 μ g/mL) (Invitrogen Corp) as supplements. HEC59 and FET cells were maintained in F12/Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. G418 sulfate (CellGro; Media Tech Inc, Herndon, VA) at 400 μ g/mL was added to cultures of HCT116+chr2 and HEC59+chr2

cells to maintain the transferred chromosome 2, which contains an antibiotic selection marker.^{22,23}

ACVR2 Genotyping

We amplified the 2 coding A₈ microsatellites of *ACVR2* in exon 3 (forward, 5'-TCTGCTTATTTATAG-GACTGATTGTG-3'; reverse, 5'-CGCTGTGTGACTTC-CATCTC-3') and exon 10 (forward, 5'-GTTGCCATT-TGAGGAGGAAA; reverse, 5'-GCATGTTTCTGCC-AATAATCTC) as previously described.² DNA was amplified in a thermocycler (MJ Research, Waltham, MA) in a reaction containing the primers, buffer, DNA template, deoxynucleotides, magnesium, and Taq polymerase. After polymerase chain reaction (PCR), the product bands were electrophoresed on a 6% polyacryl-amide gel, dried, and viewed with a phosphorimager (Molecular Dynamics, Sunnyvale, CA) for wild-type or frameshift mutated bands between the cell lines.

Transcriptional Expression of *ACVR2* and Activin A

RNA was extracted from HCT116, HCT116+ chr2, HEC59, and HEC59+chr2 cells using a TRIzol-based protocol. Briefly, 1 mL of TRIzol was added per 1×10^7 cells and incubated for 5 minutes. Then, 200 μ L of chloroform was added, followed by a centrifugation for 15 minutes with transfer of the aqueous phase to a fresh tube. RNA was precipitated using an equal amount of isopropanol, followed by ethanol washes and drying of the pellet. The concentration of RNA dissolved in DEPC-treated water was assessed using a Beckman Coulter DU640B spectrophotometer (Beckman Coulter, Fullerton, CA). We performed reverse-transcription (RT)-PCR using first an oligo dT for incubation at 37°C and then specific primers for exon 10 (forward, 5'-GTTGCCATT-TGAGGAGGAAA-3'; reverse, 5'-GCATGTTTCTGC-CAATAATCTC-3') of *ACVR2* or activin A (forward, 5'-CAGGATGCCCTTGCTTTGGCTGAGA-3'; reverse, 5'-CCCACATGAAGCTTTCTGATCGCGT-3') for PCR amplification in a thermocycler (MJ Research) in a reaction containing the primers, buffer, DNA template, deoxynucleotides, magnesium, and Taq polymerase. We subcloned the resulting complementary DNA fragments for *ACVR2* utilizing a TA cloning vector (Invitrogen Corp) as per the manufacturer's protocol. Fifty clones per cell line were then individually sequenced to determine the prevalence of mutated and wild-type *ACVR2* transcripts.

DNA Sequencing

PCR products were identified on a 2% agarose gel and DNA extracted from agarose using a gel extraction kit (Qiagen, Valencia, CA) followed by purification using Microcon YM30 columns (Millipore, Bedford, MA). The purified eluate was then used in the sequencing reaction with ABI Prism matrix (Applied Biosystems, Foster City, CA) with either the specific forward or reverse primer for exon 10 of *ACVR2* as per the manufacturer's protocol. Another purification using Centri SEP columns (Prince-ton Separations, Adelphia, NJ) was performed before direct sequencing in an ABI Automated Sequencer (Applied Biosystems).

Immunoprecipitation and Western Blotting

To detect presence of ACVR1, ACVR2, or ACVR2B in the colon cancer cell lines, we immunoprecipitated these receptors, followed by Western blotting. Briefly, HCT116, HCT116+chr2, and ACVR1/ACVR2 wild-type CAPAN-1 cells (as well as HEC59 and HEC59+chr2 cells) were grown to 60% confluency. Fresh media containing activin (25 ng/mL) was added for 4 hours. Protein lysates were obtained using 1 mL immunoprecipitation lysis buffer (2 mmol/L EDTA, 50 μ mol/L Tris, pH 7.5, 250 mmol/L NaCl, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride [PMSF], 10 μ g/mL aprotinin, 5 μ g/mL leupeptin, 10 μ g/mL soybean, 5 μ g/mL antipain, 0.05% sodium dodecyl sulfate) or hypotonic lysis buffer (10 mmol/L Tris-HCl, pH 7.5, 0.1 mol/L EDTA, 0.2% Nonidet P40, 3 mmol/L MgCl₂, 40 mmol/L NaF, 5 mmol/L glycerolphosphate, 10 μ g/mL aprotinin, 0.5 mmol/L benzimidazole, 5 μ g/mL leupeptin, 1 mmol/L sodium orthovanadate, 0.5 mmol/L PMSF). For immunoprecipitation, lysates were centrifuged for 15 minutes at 5°C and 13,000 rpm and the supernatant was quantified using spectroscopy. A total of 800 μ g of protein lysate was used along with 50 μ g of rabbit immunoglobulin G as a negative control and incubated overnight with 50 μ g of primary antibody and 5 μ L of 100 mmol/L PMSF. Fifty microliters of agarose beads (Upstate) and an additional 5 μ L PMSF were added, and the suspension was rotated for 3 hours at 4°C. The samples were then washed with PBS, electrophoresed on an 8.5% polyacrylamide loading and 3.9% stacking gel, and Western blotting performed using standard procedures using several PBS-Tween 0.1% washes with appropriate secondary antibody incubation. Blotted proteins were detected with horseradish peroxidase-linked secondary antibodies (Sigma Chemical Co, St Louis, MO) followed by enhanced chemiluminescence detection (Amersham, Little Chalfont, England).

Coimmunoprecipitation

The formation of a heterodimeric ACVR1/ACVR2 complex was assayed by coimmunoprecipitation techniques. Briefly, cells were treated for immunoprecipitation and lysed in freshly made coimmunoprecipitation buffer (2 μ g/mL leupeptin, 1 mmol/L PMSF, 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.5% Nonidet P40, 5 ng/mL pepstatin). Following overnight incubation with anti-ACVR1, Western blotting was performed with anti-ACVR2 antibodies.

Nuclear and Cytoplasmic Fractionation

Hypotonic lysis buffer (10 mmol/L Tris-HCl, pH 7.4, 3 mmol/L MgCl₂, 0.1% Nonidet P40) with added protease and phosphatase inhibitors were used to separate the nuclear-enriched fraction (pellet or P1) from the other compartments (supernatant or S1). Trafficking of pSMAD2 proteins to the nucleus after activin treatment was studied by Western blotting of nuclear and cytoplasmic fractions utilizing antibodies to SMAD2 (Santa Cruz Biotechnology) and phosphoSMAD2 (Santa Cruz Biotechnology) as well as histone H1 (Santa Cruz Biotechnology) and α -tubulin (Sigma Chemical Co) as loading controls and to ensure the purity of each fraction.

Transactivation Assay

Transient transfection of colon cancer cells with the activin specific p(ARE)₃-Luc plasmid with a vector expressing FAST1 transcription factors (generous gift from Malcolm Whitman, Harvard Medical School, Boston, MA) was performed to assess the effects of activin on transcription. FAST1 has been shown to associate with SMAD2/SMAD4, coined the activin responsive factor, and bind to an enhancer element called activin responsive element to specify developmental gene expression.²⁶ Reporter vectors (1 µg/well) and the pRL-TK vectors (20 ng/well) were transiently delivered by TransFectin (Pro-mega, Madison, WI) in 12-well plates with a 3:1 ratio of vector to DNA. Twelve to 16 hours posttransfection, cells were treated with 20 ng/mL of activin. Luciferase activity was measured by a dual-luciferase kit (Promega) 16–20 hours after the treatment, and normalization was performed using the *Renilla* luciferase activity expressed by the cotransfected pRL-TK vector.

Northern Blotting

ACVR2-mutant HCT116 cells and ACVR2-restored HCT116+chr2 cells were treated with activin A (R&D) at 25 ng/mL for 4 hours. RNA was then prepared using a TRIzol/isopropanol-based extraction. Twenty micrograms of RNA per sample was run on a 1.5% agarose gel containing formaldehyde and blotted using a standard protocol. A *c-MYC* probe was generated by enzymatic digestion from a c-MYC-ER construct (kindly provided by Bruno Amati, Palo Alto, CA) and labeled with ³²P using a random primer DNA labeling system (Invitrogen Corp). Hybridization was then performed overnight in 7% sodium dodecyl sulfate hybridization solution. The membrane was exposed to a phosphorimager screen (Molecular Dynamics/Amersham, Piscataway, NJ) after standard washes. Standardization of c-MYC transcripts/glyceralde-3-phosphate dehydrogenase transcripts was performed following a second hybridization with a PCR-generated probe using specific primers for the housekeeping gene *GAPDH* (forward, 5'-ACCACAGTCCATGC-CATCAC-3'; reverse, 5'-TCCACCACCCTGTTGCTGTA-3').

3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide Growth Assay

Cells were plated at a density of 10,000–20,000 cells/well in 48-well plates at 6 wells per treatment (control vs activin [25 ng/mL]). The compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; MP Biomedicals, Aurora, OH) was added to a concentration of 0.04 mg/mL medium, and the cells were then incubated for 3 hours. Color change indicative of metabolic activity was measured spectrophotometrically. For this, cells were stained for 3 hours with MTT, the reaction product was released by lysis with sodium do-decyl sulfate, and absorbency was detected at 570 nm using a Beckman Coulter DU640B spectrophotometer (Beckman Coulter).

ACVR2 Messenger RNA Knockdown via Specific Small Interfering RNA

Transient transfection of HCT116+chr2 colon cancer cells with specific small interfering RNA (siRNA) to ACVR2 was performed to assess for dependence of signaling and functional assays on the wild-type ACVR2 complemented HCT116+chr2 cells. One day before transfection, exponentially growing HCT116+chr2 cells were plated onto 12-well

plates at 9×10^4 cells/well. The following day, 100 $\mu\text{mol/L}$ of validated siRNA to activin A receptor type II (Ambion, Austin, TX) was transiently delivered by Lipofectamine 2000 (Invitrogen Corp) at a ratio of 1:1 of siRNA to transfection reagent in OPTI-MEM reduced serum-free media (Gibco, Carlsbad, CA). Forty-eight hours posttransfection, HCT116+chr2 cells were lysed for RNA and protein extraction or assayed for growth with activin A. *GAPDH* siRNA (Ambion) was also used as a control for specific gene knockdown.

Cell Cycle Analysis

Cell cycle analysis was performed after 24-hour treatment with 25 ng/mL of activin using a FACScan (Becton Dickinson, San Jose, CA). Briefly, cells were grown on 10-cm dishes until 50% confluent. After 24 hours of serum starvation, 10% serum was added with activin (25 ng/mL) for 48 hours. The cells were harvested using 0.05% trypsin, washed with PBS, and resuspended in 0.6 mL of PBS and 1.0 mL of 100% ethanol. The cells were fixed overnight at 4°C. The next day, the cells were centrifuged, washed in PBS, and centrifuged again for 5 minutes. The pellet was resuspended in 0.5 mL of a PBS solution containing 40 U/mL ribonuclease A and 50 $\mu\text{g/mL}$ propidium iodide. The cells were incubated at 37°C for 1 hour and then placed on ice until analysis with a Beckman Coulter Elite Flow Cytometer Multicycle (Phoenix Flow, San Diego, CA).

Migration Assay

HCT116, HEC59, HCT116+chr2, HEC59+chr2, and FET cells were each grown to 80% confluency in a 6-well dish. A wound was created crosswise using a sterile 1000- μL pipette tip, followed by a wash with $1 \times$ PBS to remove detached cells. Then, cells were treated with activin (25 ng/mL) in a series of 6 wells in serum-free media, and migration at the corresponding wound site (right above the cross) was documented using an Axiovert 2000 microscope with an AxioCAM HRC Camera (both Zeiss Microimaging, Thornwood, NY) at 0, 1, 2, 4, and 24 hours. Wound distance was then measured in the middle of the image, and the mean and standard deviation of each treatment were determined.

Transwell Migration Assay

After coating Corning Costar Transwell 24-well plates (8- μm pores; Corning, Corning, NY) with 0.1% fibronectin (Sigma Chemical Co) and blocking with 1% bovine serum albumin in $1 \times$ PBS for 1 hour at 37°C, HCT116 and HCT116+chr2 cells were seeded in triplicate at 0.5×10^5 cells per well in serum-free media containing 1% bovine serum albumin with or without activin (25 ng/mL). Cells were then allowed to migrate for 4 hours. After removal of media from both the chamber and the Transwell, and followed by 3 washes with $1 \times$ PBS, the chamber was gently wiped with a cotton swab. Migrated cells were fixed in 100% methanol for 1 hour and then allowed to air dry overnight. Cell staining was performed with a modified Giemsa stain (Sigma Chemical Co) at 1:20 for 1 hour. After carefully rinsing the Transwell and the chamber with water, images were captured using an Axiovert 2000 microscope with an Axio-CAM HRC Camera (both Zeiss Microimaging) at $10 \times$ magnification. Images were taken from 5 microscopic fields at the center of each well, and the stained cells on the bottom of the chamber were counted.

Statistical Analysis

Student *t* test was used for quantification of growth, cell cycle, and migration analysis, with a *P* value of .05 indicating statistical significance.

Results

ACVR2 Is Complemented by Stable Chromosome 2 Transfer in MSI-H, ACVR2-Mutant Cells

We tested several microsatellite stable (HCT116+ chr3, FET, SW480, HEC59+chr2) and unstable (HCT116, HCT116+chr2, HEC59) cell lines for mutations in the coding A₈ microsatellite of exon 3 and exon 10 of *ACVR2*. None of the cell lines tested revealed a frameshift in the coding polyadenine tract of exon 3 (data not shown). As expected, the mismatch repair–proficient colon cancer cell lines SW480 and FET possessed wild-type A₈ in exon 10. The mismatch repair–deficient colon cancer cell line HCT116, as well as the mismatch repair–deficient endometrial cancer line HEC59, revealed a frameshift mutation from A₈ to A₇ in exon 10 of *ACVR2*, with no wild-type A₈ present (Figure 1A). Stable transfer of chromosome 2, which harbors a single copy of wild-type *ACVR2*, restored the wild-type A₈ band in HCT116+chr2 and HEC59+chr2 cells (Figure 1A). Transfer of chromosome 3 to HCT116 cells (HCT116+chr3), which harbor the missing mismatch repair gene *hMLH1* (Table 1), did not lead to the appearance of a wild-type *ACVR2* A₈ exon 10 band. Thus, despite missing *hMLH1*, HCT116+chr2 cells contain a single copy of a stably transferred wild-type *ACVR2* in addition to 2 native mutant copies of the gene.

Complementation With Chromosome 2 Is Sufficient to Lead to Wild-Type *ACVR2* Transcript Expression

To demonstrate intact transcription from the complemented *ACVR2* gene, 50 complementary DNA clones containing the polyadenine tract within exon 10 of *ACVR2* were generated from HCT116 and HCT116+chr2 cells and sequenced. As expected, all clones from the mismatch repair–deficient and *ACVR2*-mutant HCT116 cells revealed frameshift mutations with no wild-type A₈ sequences. Complementation with chromosome 2 in HCT116+chr2 cells led to expression of the wild-type transcript in 20% of the clones despite lacking the MMR protein hMLH1 (Figure 1B). In comparison, while the mismatch repair–deficient endometrial cell line HEC59 expressed 50 transcripts with a frameshift mutation, 33% of the chromosome 2–complemented and *hMSH2*- and *ACVR2*-restored HEC59+chr2 cells expressed the wild-type A₈ tract (Figure 1B). This is at the expected ratio of 33% if transcription occurs from the single wild-type and 2 mutant *ACVR2* genes.

ACVR2 Is Expressed in HCT116+chr2 Cells and Colocalizes With ACVR1 Upon Activin Treatment

To establish expression of *ACVR2* protein following complementation with chromosome 2, we immuno-precipitated *ACVR1* and *ACVR2* in HCT116 and HCT116+chr2 cells. While both cell lines express *ACVR1*, *ACVR2B* is not expressed (data not shown) and *ACVR2* protein is only expressed in the chromosome 2–complemented HCT116+chr2 cells (Figure 2A). In our coimmunoprecipitation experiments, there appears to be some *ACVR2* associated with *ACVR1* in a preformed complex, possibly even when *ACVR2* is mutated.

This complex is further induced upon activin treatment in control CAPAN-1 cells and in *ACVR2*-complemented HCT116+chr2 cells but not in *ACVR2*-mutated HCT116 cells (Figure 2B).

SMAD2 Is Phosphorylated and Translocates to the Nucleus Following Activin Treatment in *ACVR2*-Restored Cells

To establish whether signaling is restored with *ACVR2* complementation, we prepared nuclear and cytoplasmic extracts from HCT116 cells and *ACVR2*-restored HCT116+chr2 cells following 4 hours of activin treatment. After electrophoretic separation, Western blotting was performed using SMAD2 as well as phosphoSMAD2 specific antibodies. While total SMAD2 was present in the cytosol and in the nucleus, phosphorylated SMAD2 was solely located in the nucleus. Even *ACVR2*-mutated HCT116 cells revealed a modest induction of pSMAD2 following ligand treatment (to the levels of unstimulated HCT116+chr2 cells, and independent of *ACVR2* stimulation), which was significantly pronounced after activin treatment only in the *ACVR2*-restored HCT116+chr2 cells (Figure 2C). Similarly, HEC59+chr2 cells showed a pronounced pSMAD2 induction following activin treatment from baseline nuclear presence of pSMAD2 (data not shown).

SMAD-Induced Activin-Specific Transactivation Is Restored in *ACVR2*-Complemented Cells

To assess the effect of activin on SMAD-specific transcriptional activation, we performed a reporter assay using the pARE-Luc reporter, which is indicative of activin-specific transcriptional activity.²⁶ Following 24 hours of activin treatment, there was an 11-fold increase in luciferase activity in the *ACVR2*-restored HCT116+chr2 cells compared with the *ACVR2*-mutated HCT116 cells (Figure 3A).

Activin Induces *c-MYC* Down-regulation in *ACVR2*-Restored Cells

The effect of restored *ACVR2* and SMAD signaling was assessed by Northern blotting of *c-MYC*, a known TGF- β and activin transcriptional target gene.⁴ While treatment with activin did not change *c-MYC* expression in the *ACVR2* mutant HCT116 cells, *c-MYC* was down-regulated in the *ACVR2*-complemented HCT116+chr2 cells (Figure 3B). A more pronounced decrease in *c-myc* expression was seen in the activin-responsive FET colon cancer cell line following activin treatment (data not shown).

Cell Growth Is Suppressed Following Activin Treatment in *ACVR2*-Complemented Cells

To discern whether treatment with activin induces changes in cellular growth in cancer cells with intact activin signaling, we treated HCT116, HEC59, and *ACVR2*-restored HCT116+chr2 and HEC59+chr2 cells with activin. Activin caused a decrease in growth in the *ACVR2*-complemented cell lines, but cells harboring mutations in *ACVR2* displayed no change in their growth pattern (Figure 4). The activin-sensitive FET colon cancer cells were used as positive control for comparison.

Activin A Is Expressed in ACVR2-Complemented HCT116+chr2 Cells, Demonstrating the Potential for Autocrine-Induced Growth Suppression In Vivo

Our laboratory has previously reported an in vivo determination of growth of HCT116, HCT116+chr2, and HCT116+chr3 cells in a nude mouse model.²⁷ At that time, it was not known what could modulate growth of the chromosome-complemented cell lines, because the main goal of that experiment was to determine if *hMLH1*-complemented HCT116+chr3 cells would continue to be tumorigenic. The results of the experiment showed that HCT116, HCT116+chr2, and HCT116+chr3 cells were tumorigenic in mice, with measurable tumors detected by days 6–7 in HCT116-injected mice and tumors detected at days 8–9 for HCT116+chr2- and HCT116+chr3-injected mice.²⁷ However, both HCT116+chr2 and HCT116+chr3 cells produced tumors that grew at a significantly lower rate than the parental cell line HCT116. We now believe that restored activin signaling in ACVR2-complemented HCT116+chr2 cells and restored TGF- β signaling in *TGFBR2*-complemented HCT116+chr3 cells are at least partially responsible for their reduced growth when compared with parental HCT116 cells (Table 1). To ascertain whether this hypothesis is a potential mechanism for reduced growth in HCT116+chr2 cells, we assessed if there could be autocrine production of activin A in HCT116+chr2 cells. We amplified *activin A* messenger RNA using semiquantitative RT-PCR. Both HCT116 and HCT116+chr2 cells expressed activin A ligand (Figure 5), but the effects of activin A would only be expected to be realized in the ACVR2-restored HCT116+chr2 cells.

Use of ACVR2-Specific siRNA in HCT116+chr2 Cells Shows That Activin-Induced Signaling and Growth Suppression Are ACVR2 Dependent

To establish that the effects of activin observed in HCT116+chr2 cells are specific to ACVR2 complementation and not other genes located on chromosome 2, we performed knockdown experiments using ACVR2-specific siRNA (Figure 6). We were able to knock down ACVR2 transcripts to none-detectable levels by RT-PCR techniques with the ACVR2-specific siRNA, which was more effective than our knockdown of the housekeeping gene messenger RNA of *GAPDH* (Figure 6A). We then assessed the ACVR2 siRNA knockdown HCT116+chr2 cells for phosphoSMAD2 signaling as well as activin-induced growth suppression. Following knockdown of ACVR2, activin-induced phosphoSMAD2 activation and nuclear translocation were muted when compared with mock siRNA-transfected HCT116+chr2 cells treated with activin (Figure 6B). Furthermore, knockdown of ACVR2 in ACVR2-complemented HCT116+chr2 cells prohibited activin-induced growth suppression, completely reversing the effects seen in mock siRNA-transfected HCT116+chr2 cells (Figure 6C). Thus, in addition to the activin-specific signaling and growth suppression seen in ACVR2-complemented HCT116+chr2 cells, siRNA knockdown of the complemented ACVR2 gene abrogated the effects.

Activin Decreases the S-phase Cell Cycle Fraction in ACVR2-Restored Cells

To determine the effects of activin on the cell cycle, we studied cell cycle distribution of ACVR2-deficient HCT116 cells and ACVR2-restored HCT116+chr2 cells following activin treatment. While there was no change in the proportions of cells in G₁, S, and G₂ phase in ACVR2-mutant cells following activin treatment, ACVR2-restored cells displayed a decrease

in S-phase following activin treatment. (Figure 7A). In *ACVR2*-restored HEC59+chr2 cells, a concomitant increase in G₁ phase was observed (Figure 7B).

Intact Activin Signaling Enhances Migration in Colon Cancer Cells

To assess a potential effect on cellular migration with activin signaling akin to that observed for TGF- β , we treated *ACVR2*-mutant HCT116 and *ACVR2*-restored HCT116+chr2 cells with activin in a cellular wound assay and a Transwell migration assay. While HCT116 cells did not display enhanced migration across a wound in response to activin, cell motility of HCT116+chr2 cells was markedly enhanced over *ACVR2*-mutated control cells (Figure 8A and B) and became evident as early as 1 hour after activin treatment. Similarly, *ACVR2*-restored HCT116+chr2 cells had significantly more cells that migrated through pores in a Transwell in response to activin than *ACVR2*-mutant HCT116 cells (Figure 8C). There was an upward trend that did not reach significance when comparing untreated HCT116 with untreated HCT116+chr2 cells, which might be reflective of autocrine activin signaling with a restored pathway. Thus, both the cellular wound assay and the Transwell migration assay indicate that activin increases cell migration when activin signaling is intact.

Discussion

The biological effects of activin in colon cancer have not been previously appreciated and recently came to light when the *ACVR2* receptor was discovered to be mutated in the majority of MSI-H colon cancers.^{2,20} Here, we demonstrated a novel model system in which a single physiologically regulated copy of *ACVR2* was complemented in MSI-H colon cancer cells. We found that the classic activin-SMAD signaling pathway is restored, is transcriptionally responsive, and, importantly, induces growth suppression. Although not the major focus of this study, activin appears to be growth suppressive in *ACVR2*-restored endometrial cancer cells as well. These findings underscore the role of activin in braking cell growth in these colon cancer and endometrial cancer cells.

We found that activin signaling is reconstituted in MSI colon cancer cell lines with one copy of wild-type *ACVR2*. We further showed that *ACVR2*/*ACVR1* exists in a pre-formed complex, possibly even if *ACVR2* is expressed in its truncated form. This is in accordance with the previous observation that only mutations that involve the activin-binding site of *ACVR2* located in the extracellular domain result in loss of *ACVR1* binding.²⁸ Further, truncated type 2 and type 1 receptors that lack their entire intracellular domain were found to cross-link to the ligand in a cooperative manner.²⁹ Frameshift mutation of exon 10 is downstream of the extracellular domain and affects the cytosolic domain of the receptor, therefore maintaining its ability to bind ligand and form a heteromeric complex with *ACVR1*. It is also possible that, with *ACVR2* restoration, some activin signaling through the *ACVR2*/*ACVR1* complex occurs from autocrine activin A production. Indeed, in *ACVR2*-complemented HCT116+chr2 cells, activin A is transcribed, the cells have some pre-formed *ACVR2*/*ACVR1* complexes, and there is a minimal nonstimulated amount of phosphoSMAD2 present on Western blots. Additional *ACVR2*/*ACVR1* complex induction occurs in the *ACVR2*-restored colon cancer cells following ligand binding, likely

representing recruitment of additional ACVR1 receptors and resulting in enhanced pSMAD2 activation.

Activin has been shown to regulate growth in myeloid and hematopoietic cells,^{8,9} but there are limited data in epithelial cells, particularly colon cancer cells. In breast cancer cells, activin is growth suppressive,⁷ similar to what we found in our reconstituted colon cancer cell model. While our model has the advantage of being physiologic, with only one copy of ACVR2 restored as opposed to an overexpression model, this may have led to modest growth effects. The fact that we compared cells with and without ligand in multiple clones of 2 different models implicates ACVR2 as the target on chromosome 2. This is further confirmed by our ACVR2-selective knockdown experiments, which showed a reversal of the activin-induced signaling and growth suppression. The mechanism of growth suppression, however, is not entirely clear. Antagonism of c-MYC is implicated because c-MYC is down-regulated by activin in prostate cancer cells⁴; in keratinocytes, Mad1, a negative regulator of c-MYC, is induced.¹¹ We found a down-regulation of c-MYC in our ACVR2-restored model, indicating c-MYC may be regulated by activin in colon cancer cells as well. Cellular growth clearly appears to be regulated by activin-induced SMAD-dependent signaling because cells mutant for ACVR2 do not show response to activin. Both pituitary and pancreatic tumors harbor loss-of-function mutations in activin signaling components^{16,17} and, with our current data, underscore the role of activin signaling in tumor suppressor suppression. Intact activin signaling also appears to reduce the size and volume of colon cancers.³⁰

In MSI-H colon cancers, both TGF- β and activin are abrogated due to frameshift mutations in their type II receptor.² The loss of both of these signaling pathways must be beneficial for tumor growth. Both ACVR2-restored and TGFBR2-restored colon cancer cells are tumorigenic in nude mice, but both complemented cells grow slower than its parental counterpart.²⁷ Both TGF- β and activin use the same intracellular SMAD proteins (SMAD 2 and 3) to transmit their signal to the nucleus. It is not clear to date how each respective ligand differentiates its signal through these identical SMADs, but we speculate that both ligands may have an additive effect in their growth response because both are inactivated in MSI-H colon cancers.

While ligands from the TGF- β family, such as TGF- β , activin, and the BMPs, are believed to signal in a similar manner, the activin/ACVR2 interface is distinct from the TGF- β /TGFBR2 interface,²⁹ possibly alluding to functional differences. This is further underscored by the finding that ACVR2 regulates many genes related to the TGF- β pathway as well as some genes that have not been associated with TGF- β signaling.³¹

Despite the growth suppression observed in our ACVR2-restored cellular models, activin enhances cellular motility. Our finding is congruent with the fact that migration of normal colon epithelial cells is enhanced by TGF- β ² and with the recent observation of somatic acquisition of activating mutations in TGFBR1 in metastases and not from the source of primary colon cancers,³³ as well as worse survival with wild-type TGFBR2 in patients with MSI-H colon cancers.³⁴ Additionally, the non-invasive activin-sensitive colon cancer cell line FET did not show enhanced migration with activin treatment (data not shown), while the complemented HCT116+chr2 colon cancer cells, which are invasive,³⁵ did.

In contrast to an *ACVR2*-overexpression model,³¹ complemented HCT116+chr2 cells produce physiologic levels of *ACVR2* and remain tumorigenic in nude mice,²⁷ indicating that the presence of wild-type *ACVR2* does not abrogate tumor growth and invasion in these cells. However, when compared with parental HCT116 cells, mean tumor volume was significantly less in the HCT116+chr2 cells, which can now be explained by the complementation of *ACVR2* in conjunction with activin A production.²⁷ Furthermore, neuroblastoma cells with restored activin expression exhibit diminished proliferation but an unchanged lung metastasis rate,³⁶ and invasion is enhanced by activin in ovarian cancer cell lines.³⁷ Taken together, activin may promote cell migration and possibly contribute to the metastatic process.

In conclusion, our single-copy *ACVR2*-complemented MSI-H cell models revealed normal expression of *ACVR2* and induced activin-specific transcriptional responses, with the biologic response of growth suppression, slower S-phase cycling, and enhanced migration. These responses are similar to that of intact TGF- β signaling in colon cancer, and loss of these activin responses contributes to the pathogenesis of MSI-H colon cancer.

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Abbreviations used in this paper

MSI-H	high-frequency microsatellite instability
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PMSF	phenylmethylsulfonyl fluoride
RT-PCR	reverse-transcription polymerase chain reaction
siRNA	small interfering RNA
TGF	transforming growth factor

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Figure 1.

Chromosome 2 complementation shows the presence of the wild-type *ACVR2* allele and leads to expression of wild-type transcripts in HCT116+chr2 and HEC59+chr2 cells. (A) Frameshift mutation of exon 10 of *ACVR2* was assessed by amplification of the DNA of the cell line using ³²P-labeled primers. Note acquirement of the wild-type A₈ allele in chromosome 2–complemented cells. FET and SW480 are microsatellite stable colon cancer cell lines. (B) RNA from each cell line was reverse transcribed, and the complementary DNAs were sub-cloned and sequenced to assess for *ACVR2* expression. Note the acquisition of wild-type A₈ transcripts (or T₈ on the reverse strand) with chromosome 2 complementation.

Figure 2.

Complemented *ACVR2* causes expression of *ACVR2* protein that complexes with *ACVR1* with activin stimulation and phosphorylates *SMAD2* for its nuclear translocation. (A) Immunoprecipitated *ACVR1* protein is present in parental and chromosome 2–complemented cells, but immunoprecipitated *ACVR2* protein is only present in chromosome 2–complemented cells. (B) *ACVR2* protein coimmunoprecipitated with *ACVR1* antibody in HCT116+chr2 and control CAPAN cells, but not in *ACVR2*-mutant HCT116 cells. Activin stimulation further increased *ACVR1/ACVR2* complex formation. (C) *SMAD2* is phosphorylated after activin stimulation minimally in HCT116 cells but is markedly activated in *ACVR2*-complemented HCT116+chr2 cells and translocates into the nucleus. Histone and tubulin blots were performed to indicate the relative purity of the nuclear and cytoplasmic fractions, respectively.

Figure 3.

Chromosome 2 complementation restores activin-induced transactivation. (A) The pARE-Luc reporter was transfected into cells before stimulation with activin. (B) Northern blotting for c-Myc expression, known to be down-regulated by activin, was performed using a generated c-Myc probe. A GAPDH probe was used as a loading control, and the relative expression of c-Myc to GAPDH is shown in the bar graph.

Figure 4.

Activin is growth suppressive in chromosome 2–complemented cells. The MTT assay, which approximates cell growth, demonstrates a reduction of growth with activin in the chromosome 2–complemented cells only. Activin-responsive FET colon cancer cells were used as a positive control (* $P < .05$).

Figure 5.

Both HCT116 and HCT116+chr2 cells transcribe native activin A ligand. RT-PCR was performed on extracted RNA from the cells utilizing specific activin A oligonucleotides for amplification. GAPDH was used as a loading control.

Figure 6.

ACVR2 silencing reverses activin signaling and growth effects observed in the *ACVR2*-complemented HCT116+chr2 cells. (A) *ACVR2* messenger RNA expression is knocked down in *ACVR2*-complemented HCT116+chr2 colon cancer cells using *ACVR2*-specific siRNA. RT-PCR followed by agarose gel electrophoresis show the specificity of siRNA to knock down *GAPDH* and *ACVR2* mRNA. dH₂O, distilled water only; C, control; S, scrambled siRNA; KD, gene-specific knockdown via siRNA treatment. (B) *ACVR2* knockdown abolished activin-induced pSMAD2 activation and its nuclear translocation in *ACVR2*-complemented HCT116+chr2 cells. KD, *ACVR2*-specific siRNA knockdown;

Mock, treatment without any specific siRNA. Histone and tubulin Western blots were performed to indicate the relative purity of the nuclear and cytoplasmic fractions, respectively. (C) Activin-induced growth suppression is abrogated by *ACVR2* knockdown in *ACVR2*-complemented cells by the MTT assay (* $P < .05$).

Figure 7. Activin slows the S phase in chromosome 2–complemented cells. Cell cycle analysis revealed no changes after activin treatment without chromosome 2 complementation. (A) HCT116 and HCT116+chr2 cells. (B) HEC59 and HEC59+ chr2 cells. (* $P < .05$).

Figure 8.

Activin enhances cell migration in chromosome 2-complemented cells. (A)

Photomicrographs from the wound assay are shown. (B) Graphical depiction of the data from the photomicrographs in A. (C) Data from a Transwell migration assay (8- μ m pore size) are shown. Note that activin increased migration in both the wound assay and the Transwell migration assay (* $P < .05$).

Table 1

Cell Lines Used in This Study

Cell line	Key affected genes	Key restored genes
HCT116	<i>ACVR2, TGFB2, hMLH1</i>	—
HCT116+chr2	<i>TGFB2, hMLH1</i>	<i>ACVR2</i>
HCT116+chr3	<i>ACVR2</i>	<i>TGFB2, hMLH1</i>
FET	None	—
SW480	<i>SMAD4</i>	—
HEC59	<i>hMSH2, ACVR2, TGFB2</i>	—
HEC59+chr2	<i>TGFB2</i>	<i>ACVR2, hMSH2</i>