

Oncogenic β -Catenin Is Required for Bone Morphogenetic Protein 4 Expression in Human Cancer Cells¹

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Abstract

Somatic cell gene targeting was used to create an isogenic set of human colon cancer cells that differs only in the presence or absence of their endogenous activated β -catenin oncogene. Affymetrix Genechip expression profiling of parental cells and gene-targeted derivatives identified numerous novel genes whose expression was dependent on the presence of oncogenic β -catenin. The transforming growth factor- β family member bone morphogenetic protein 4 (BMP4), whose receptor is mutated in a rare inherited gastrointestinal cancer predisposition syndrome, was the most highly differentially expressed gene. Additional experiments revealed that the oncogenic allele of β -catenin specifically is absolutely required for BMP4 expression and secretion by human cancer cells and that BMP4 is overexpressed and secreted by human colon cancer cells with mutant adenomatous polyposis coli genes. These data identify the presence of regulatory interactions between the Wnt and BMP signaling pathways in cancer pathogenesis, providing an intriguing connection between the sporadic and inherited forms of a common human malignancy.

Introduction

Activation of the β -catenin oncogene is a common event in the pathogenesis of a wide range of human tumors (1). Oncogenic β -catenin serves as a transcription factor effector in the Wnt signaling pathway by heterodimerizing with DNA-binding proteins known as TCFs³ and providing a transcriptional activation domain (2). Several putative β -catenin target genes have been identified; however, identification of effectors of the β -catenin oncogene has been complicated by the paucity of suitable experimental systems. In an effort to create an experimental system well suited for identification of such effectors, we chose human somatic cell gene targeting. Such an approach is ideal for studying the function of heterozygous-activated oncogenes, because it allows investigators to specifically delete either the mutant, activated allele or the wild-type allele of an oncogene. The resulting isogenic set of human cancer cells (composed of parental cells with both the wild-type and mutant, activated allele of the gene and derivatives in which either the wild-type or mutant allele of the gene has been deleted) provides a powerful experimental system for studying oncogene biology because it is based on naturally occurring human cancer cells and does not use ectopic overexpression of the

oncogene. Here, we describe the use of such an approach to generate an isogenic system of human cancer cells that differ only in the presence or absence of their endogenous β -catenin oncogene and its use in identifying putative effectors of oncogenic β -catenin. We identify *BMP4* as a gene whose regulation is strikingly dependent on the presence of activated β -catenin.

Materials and Methods

Creation of a Human β -Catenin Targeting Vector. To identify genomic clones corresponding to the human β -catenin gene, a commercially available bacterial artificial chromosome library was screened with a radiolabeled, PCR-generated probe consisting of β -catenin exons II–V (Research Genetics, Huntsville, AL; Ref. 3). Four bacterial artificial chromosomes were identified (CITB 49D12, 79O13, 99F24, and 591L10). To create a restriction map of the cloned genomic region, Southern blots were performed using radiolabeled, exon-derived oligonucleotides as probes. A combination of shotgun cloning and PCR was then used to subclone a 9.8-kb contig containing exons II–VI. The subcloned region was then sequenced in its entirety (GenBank accession no. AY081165). The sequence was analyzed with Lasergene software (DNAS-TAR) and with Repeatmasker⁴ to create a sequence-based restriction map and identify the locations of repeat elements, respectively. An 8.9-kb segment of subcloned genomic DNA was then subcloned into yeast shuttle vector pRS425 to make pRS425- β -Cat.

Homologous recombination in *Saccharomyces cerevisiae* was then used to replace β -catenin exons II–IV with a neo^R gene, precisely substituting the initiating methionine of β -catenin with that of the neo^R gene. To do this, pRS425- β -Cat was linearized in β -catenin exon IV (*Xho*I). High-fidelity PCR was used to create a neo^R gene with various sequence features on each end. These included 50 nucleotides of homology to β -catenin exons II and IV for directing a specific recombination event with the subcloned β -catenin genomic region and a PCR priming site to facilitate eventual identification of gene-targeted human cells (Fig. 1A). The linearized shuttle vector and the PCR-generated neo^R gene were cotransformed into *S. cerevisiae*. Leu⁺ colonies were screened by PCR and sequencing to identify and characterize recombinants. The targeting vector was then shuttled into *Escherichia coli*, and its integrity was confirmed via restriction analysis and resequencing of critical junctions. The specific details of construction and the sequences of all PCR primers used are available from the authors on request.

Tissue Culture and Transfection. HCT116, SW480, HT29, LoVo, DLD1, and 293 cells (American Type Culture Collection, Manassas, VA) were grown at 37°C in 5% CO₂ in media containing 10% fetal bovine serum (Mediatech, Herndon, VA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). HCT116 cells; gene-targeted HCT116 derivatives; and DLD1, HT29, and 293 cells were grown in McCoy's 5A media (Invitrogen), LoVo cells in Ham's F12 media (Invitrogen), and SW480 cells in Lebovitz's L15 media (Invitrogen). HCT116 cells were transfected with linearized β -catenin targeting vector (*Not*I) using Lipofectamine (Invitrogen), following the manufacturer's protocol. To obtain stably transfected clones by limiting dilution, cells were trypsinized ~18 h after transfection, mixed with media containing 0.6 mg/ml G418 (Invitrogen), and distributed to 96-well plates. After ~2 weeks of growth, individual colonies were transferred first to wells in a 24-well plate, then to a 25-cm² flask. Cells in confluent flasks were trypsinized, and approx-

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³ The abbreviations are: TCF, T-cell factor; BMP, bone morphogenetic protein; SAGE, serial analysis of gene expression; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; APC, adenomatous polyposis coli; TGF, transforming growth factor.

⁴ Internet address: <http://repeatmasker.genome.washington.edu>.

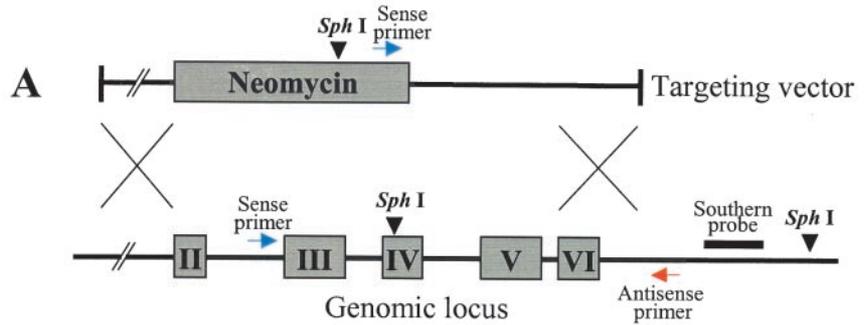
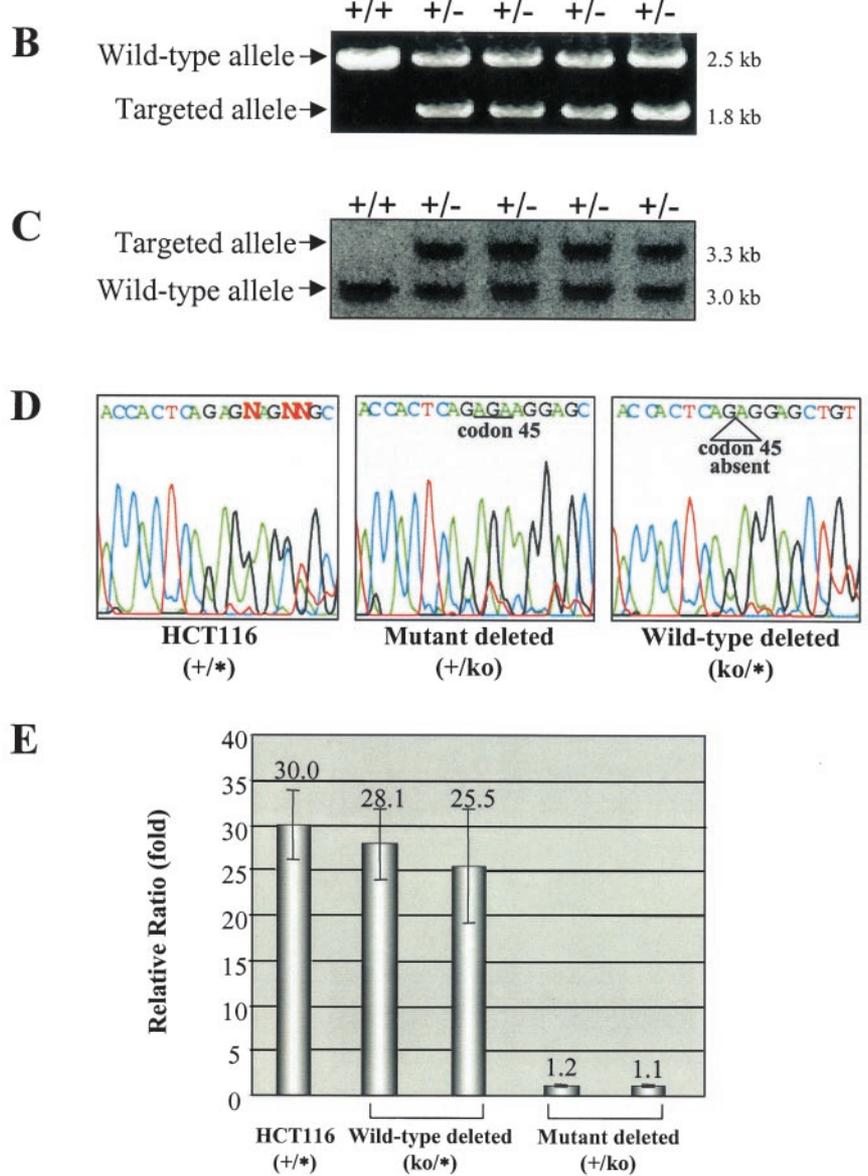


Fig. 1. Gene targeting of the human β -catenin oncogene. **A**, alignment of the human β -catenin genomic locus with the targeting vector. *Gray boxes*, exons II–VI. Homologous recombination between the genomic locus and the targeting vector deletes exons II–IV and replaces them with a promoterless neo^R gene. PCR primers used for identification of knockouts are indicated, as are the restriction enzyme cleavage sites and probe used for Southern blot-based confirmation of knockouts. **B**, PCR-based identification of gene-targeted clones. Parental HCT116 cells have a single 2.5-kb band derived from two untargeted alleles of β -catenin. Heterozygous knockout clones have a 2.5-kb band derived from the untargeted allele and a 1.8-kb band from the targeted allele. PCR priming sites are depicted in **A**. **C**, Southern blot-based confirmation of gene-targeted clones. Parental HCT116 cells have a single 3-kb band derived from two untargeted alleles of β -catenin. Heterozygous knockout clones have a 3-kb allele derived from the untargeted allele and a 3.3-kb band from the targeted allele. The locations of the restriction sites and probe are depicted in **A**. **D**, sequence-based identification of the untargeted allele. PCR products from the untargeted allele(s) were sequenced with an antisense primer to determine whether the untargeted allele was wild type (+) or mutant (*). In parental HCT116 cells (+/*), the sequence diverges at serine 45 because the sequencing template is a mixture of wild-type and mutant β -catenin genes. Gene-targeted clones in which the wild-type allele remained are depicted as (+/ko). Gene-targeted clones in which the mutant, oncogenic allele remained are depicted as (ko/*). **E**, functional analysis of gene-targeted cells. HCT116 cells of various β -catenin genotypes were transfected transiently with firefly luciferase reporters (OT-TOPFLASH or OF-FOPFLASH) to measure TCF activity and a Renilla luciferase expression vector as an internal control for transfection efficiency. Results are depicted as a ratio of normalized OT activity (with wild-type, TCF-binding sites) to normalized OF activity (with mutant TCF-binding sites). The experiment was repeated in triplicate on 3 different days. Mean values are indicated above each bar. *Bars*, SE.



imately one-third of the cells was used for preparation of genomic DNA, and two-thirds were used for cryopreservation.

Genomic PCR, Southern Blots, and DNA Sequencing. Preparation of genomic DNA, PCR, Southern blots, and automated sequencing were all performed using standard techniques. Taq Platinum (Invitrogen) was used for PCR, according to the manufacturer's instructions. For Southern blots, 5 μ g of genomic DNA were digested with *Sph*I, separated on a 1% agarose gel, and transferred to a Zeta-Probe nylon membrane (Bio-Rad, Hercules, CA). The

membrane was prehybridized overnight at 60°C, then hybridized for 24 h at 60°C using a radiolabeled, PCR-generated probe for β -catenin intron VI. The blot was then washed and imaged using a Molecular Dynamics phosphor-imager.

Luciferase Assays. TCF reporter assays were conducted with OT-TOPFLASH/OFF-FOPFLASH (generously provided by Bert Vogelstein, Johns Hopkins School of Medicine, Baltimore, MD; Ref. 4). Confluent HCT116 cells and gene-targeted derivatives were passaged 1:4 into the wells of a six-well plate.

Approximately 24 h after plating, the cells were transfected with 600 ng of firefly luciferase reporter plasmids and 12 ng of tkRenilla (Promega, Madison, WI) as an internal control for transfection efficiency. The cells were washed once with PBS and harvested in 500 μ l of $1 \times$ passive lysis buffer at 24 h after transfection. Firefly luciferase and Renilla luciferase activities were measured with commercially available luciferase substrates (Promega), as recommended by the manufacturer.

Microarrays. Total RNA from HCT116 cells and two independently derived, gene-targeted derivatives lacking the β -catenin oncogene were prepared with TRIZOL Reagent (Invitrogen). The cRNA probes were prepared and hybridized to an U95A Genechip (Affymetrix, Santa Clara, CA), as recommended by the manufacturer. The hybridized array was stained with streptavidin-phycoerythrin conjugates and scanned using a Hewlett Packard Gene-Array scanner. The signal intensities for the β -actin and GAPDH genes were used as internal quality controls. The ratio of fluorescent intensities for the 5' end and the 3' end of these housekeeping genes was <2 . Scanned images were analyzed with Microarray Suite 4.0 software (Affymetrix). No normalization was performed because the average intensities of the Genechips differed by less than 10%. Genes identified as absent or whose maximum expression was <200 in all three samples were excluded from the analysis. All expression intensities <50 were set to 50, and the fold change of a particular gene was determined as the average of fold changes between expression levels of the gene in HCT116 cells and in each of the gene-targeted derivatives.

Northern Blots. Total RNA from HCT116 cells and gene-targeted derivatives was prepared with TRIZOL Reagent (Invitrogen) according to the manufacturer's instructions. PolyA RNA was purified using the MessageMaker System (Invitrogen) as recommended by the manufacturer. PolyA RNA (10 μ g) was subjected to agarose/formaldehyde gel electrophoresis. After staining and removal of formaldehyde, the RNA was transferred to a Zeta-Probe nylon membrane (Bio-Rad). The membrane was prehybridized in QuikHyb (Stratagene, La Jolla, CA) overnight at 68°C, then hybridized for 24 h at 68°C in QuikHyb containing a radiolabeled, PCR-generated DNA probe for the human *BMP4* gene or the human *GAPDH* gene. The membrane was then washed and imaged using a phosphorimager (Molecular Dynamics). Band intensities were quantified using ImageQuant software (Molecular Dynamics).

BMP4 ELISA. Equivalent numbers of human cells were plated in 25-cm² flasks and allowed to grow to confluence (generally 2–3 days). The media were then changed to 2.5 ml of serum-free media and allowed to incubate on the cells for 48 h. The cells remained healthy during the conditioning period, despite the absence of serum. The conditioned media were then removed, centrifuged to remove any floating cells, and tested for the presence of BMP4 using a commercially available BMP4 ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Dilutions of purified BMP4 protein standard were used to calibrate the assay.

Results

Heterozygous β -Catenin Gene Targeting in Human Cancer Cells. HCT116 human colon cancer cells were chosen for β -catenin gene targeting because they contain a heterozygous-activating mutation in β -catenin (in-frame deletion of serine 45) and are well established as suitable for human somatic cell gene targeting (5, 6). The human promoterless β -catenin targeting vector was created as described in "Materials and Methods." It was designed to delete the first three coding exons of β -catenin and precisely replace them with a promoterless neo^R gene.

To create gene-targeted clones, HCT116 cells were transfected with linearized β -catenin targeting vector and individual drug-resistant clones isolated by limiting dilution. A group of 216 individual clones was obtained. Each clone was cryopreserved and used for the preparation of genomic DNA.

A PCR-based strategy was used to identify clones in which the targeting vector had integrated by homologous recombination. The sense primer (5'-TTTCATCACTGAGCTAACC-3') was located in intron II, and the antisense primer (5'-CTGGCACTTAACCTCATC-3') was located in intron VI, just outside the right homology arm of the targeting vector. The targeting vector was designed such

that homologous integration would alter the size of the PCR product by deleting the endogenous intron II priming site and moving it 0.7 kb downstream (Fig. 1, A and B). As such, PCR products derived from wild-type β -catenin alleles were predicted to be 2.5 kb, and PCR products generated from targeted alleles were predicted to be 1.8 kb. This recently developed PCR strategy was chosen because it provides an internal positive control in each PCR reaction without multiplexing (7). Twenty-six of the 216 clones were knockouts, a targeting frequency of 12%.

A Southern blot strategy was used to further confirm the knockouts, using *SphI* and a PCR-generated, radiolabeled probe located just downstream of the right homology arm. Wild-type alleles were predicted to be 3 kb, and targeted alleles were predicted to be 3.3 kb (Fig. 1C). As expected, parental HCT116 cells contained only wild-type alleles, and β -catenin gene-targeted derivatives contained both wild-type and targeted alleles.

To determine which allele of β -catenin had been deleted, PCR products derived from the untargeted allele were sequenced. Because the sequencing primer (5'-TGTTCTTGAGTGAAGGACTG-3') was located in the region deleted by the targeting vector, only the untargeted allele was sequenced. The wild-type allele remained in 58% of the clones, and the mutant (oncogenic) allele remained in 42% of the clones. Examples are shown in Fig. 1D.

Endogenous-activated β -Catenin Is Required for TCF Reporter Activation. Oncogenic β -catenin is known to bind to members of the TCF family of transcription factors and provide a transcriptional activation domain, enabling the activation of TCF-responsive reporters. As such, TCF reporter assays were performed to provide functional verification of the genotypes of the HCT116 parental cells and gene-targeted derivatives and to test whether endogenous activated β -catenin is necessary for TCF-mediated transcriptional activation. As depicted in Fig. 1E, a TCF-responsive reporter was strongly activated in HCT116 parental cells (+/*). Reporter activity was decreased slightly in gene-targeted cells with only oncogenic β -catenin (ko/*). As expected, reporter activity was completely abolished in gene-targeted cells with only wild-type β -catenin (+/ko). Reporter activity was restored by cotransfection of an activated β -catenin expression vector (data not shown; Ref. 8). Similar results were obtained using other TCF-responsive reporters as well (data not shown). These results provided independent confirmation of the genotypes described above and demonstrate that activated β -catenin is absolutely necessary for TCF-mediated transcriptional activation in human cancer cells.

Identification of β -Catenin-regulated Genes. Affymetrix U95A Genechips (containing probes for approximately 9,000 human genes) were used to identify genes more highly expressed in HCT116 cells than in gene-targeted derivatives lacking the oncogenic allele of β -catenin. Two gene-targeted clones of identical genotype were studied to reduce the potential for identifying clone-specific artifacts. The gene expression profiles were remarkably uniform between the three samples, with correlation coefficients between pairs of samples > 0.967 . Genes whose expression was ≥ 3 -fold higher in HCT116 cells than in each of the gene-targeted clones are depicted in Table 1. The gene encoding BMP4 was expressed ~ 15 -fold more highly in parental HCT116 cells than in either of the gene-targeted derivatives lacking oncogenic β -catenin. The expression difference was detected by probe sets for both the *BMP4* and *BMP2B* genes, two different identifiers for the same gene (*BMP2B*; data not shown). Other intriguing genes include a putative methyltransferase, several genes involved in immune system function, and motor proteins involved in cytokinesis.

Next, publicly available data generated by SAGE was mined to narrow down the list by determining which of the genes were simi-

Table 1 β-catenin regulated genes

	Gene	Locus ID	Function	Fold change
1	BMP4	652	Development/differentiation	14.6
2	RAD23 homolog A	5886	DNA repair	8.2
3	Phosphodiesterase 4B	5142	Enzyme	7.9
4	Putative methyltransferase	54743	Enzyme	5.5
5	Phosphatidylinositol glycan, class C	5279	Enzyme	5.4
6	Killer cell lectin-like receptor subfamily C, member 3	3823	Signal transduction	5.2
7	Neuregulin 1	3084	Growth factor	4.9
8	Kinesin-like 2	3833	Cytokinesis	4.9
9	Interleukin enhancer-binding factor 3 (ILF3)	3609	Transcription factor	4.8
10	Protein phosphatase 3 catalytic subunit α (PPP3CA)	5530	Enzyme	4.5
11	NB thymosin β	11013	Structural protein	4.4
12	Mitotic kinesin-like protein 1	9493	Cytokinesis	4.2

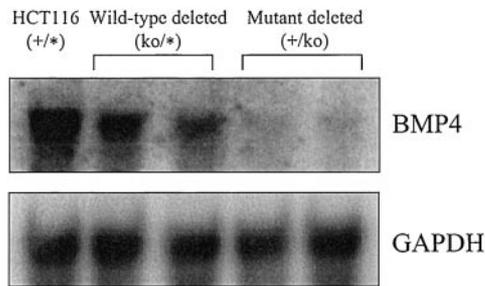


Fig. 2. Oncogenic β-catenin is required for BMP4 expression. Northern blot analysis of BMP4 expression. PolyA RNA (10 μg) from HCT116 cells and gene-targeted derivatives were separated by agarose gel electrophoresis, transferred to a nylon membrane, and analyzed with radiolabeled DNA probes for BMP4 and GAPDH.

larly differentially expressed between normal colonic epithelial cells and both HCT116 cells and SW837 cells, a human colon cancer cell line with activated β-catenin by virtue of inactivating mutations in the APC tumor suppressor gene⁵ (9). Of those genes for which SAGE data were available, the expression difference for BMP4 was by far the most consistent and impressive. BMP4 was expressed ~21-fold more highly in both HCT116 and SW837 cells (381 and 409 tags/million, respectively) than in normal colonic mucosa (19 tags/million).

On the basis of: (a) the magnitude and consistency of the Affymetrix Genechip and SAGE data; (b) the potential biological ramifications of a linkage between Wnt and BMP signaling in human cancer pathogenesis; and (c) the recent report of BMP4 receptor mutations in a rare gastrointestinal cancer predisposition syndrome (10), we decided to focus our efforts on better characterizing the regulation of BMP4 by oncogenic β-catenin in human cancer.

Oncogenic β-Catenin Is Required for BMP4 Expression.

Northern blots were performed to confirm the BMP4 expression difference and determine whether it was specific for deletion of the mutant, oncogenic allele of β-catenin. BMP4 expression was measured using polyA RNA derived from HCT116 parental cells and two independently derived gene-targeted clones of each genotype, using a PCR-generated, radiolabeled probe specific for human BMP4 (Fig. 2). Expression of BMP4 was highest in HCT116 parental cells. BMP4 expression was reduced ~30% in HCT116 cells in which the wild-type allele of β-catenin had been deleted. This result is consistent with the TCF reporter assays described above and suggests an unexpected synergy between the oncogenic and wild-type forms of β-catenin protein. Strikingly, expression of BMP4 was virtually extinguished in HCT116 cells in which the oncogenic allele of β-catenin had been deleted.

Oncogenic β-Catenin Is Required for BMP4 Secretion. To further confirm the dependence of BMP4 expression on the presence of

oncogenic β-catenin, a BMP4 ELISA was used to measure levels of BMP4 protein in conditioned media from parental HCT116 cells and gene-targeted derivatives. Protein levels were measured in two independently derived gene-targeted clones of each genotype (Fig. 3A). Of the isogenic cell lines, parental HCT116 cells (+/*) secreted the highest levels of BMP4. Gene-targeted HCT116 cells lacking the wild-type allele of β-catenin (ko/*) secreted intermediate levels of BMP4. No secreted BMP4 was detected in HCT116 cells lacking the oncogenic allele of β-catenin (+/ko).

BMP4 Overexpression in Human Colon Cancer Cells with Mutant APC Genes. If expression of BMP4 is dependent on the presence of oncogenic β-catenin, one might expect that cancer cells with activated β-catenin would uniformly overexpress BMP4. Human colon cancer is an ideal system in which to test this hypothesis,

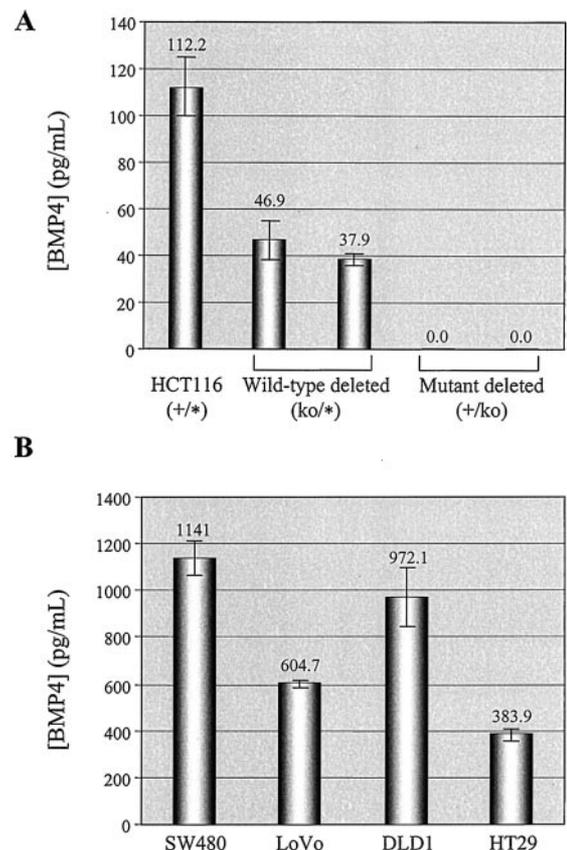


Fig. 3. BMP4 secretion in human colon cancer cell lines. ELISA analysis of secreted BMP4 protein. Serum-free media were conditioned on confluent monolayers of HCT116 parental cells and gene-targeted derivatives for 48 h (A) and confluent monolayers of SW480, LoVo, DLD1, and HT29 cells for 42 h (B). Levels of BMP4 in the conditioned media were measured by ELISA as described in "Materials and Methods." The experiment was repeated in triplicate. Mean values are indicated above each bar. Bars, SD.

⁵ Internet address: <http://www.ncbi.nlm.nih.gov/SAGE/>.

because virtually all human colon cancers have activated β -catenin by virtue of APC or β -catenin gene mutations (11). Therefore, BMP4 expression was measured in human colon cancer cell lines using a BMP4 ELISA assay.

Levels of secreted BMP4 were measured in four colon cancer cell lines with well-characterized APC gene mutations and concomitant activation of β -catenin: (a) SW480; (b) HT29; (c) DLD1; and (d) LoVo (11). As shown in Fig. 3B, each cell line secretes high levels of BMP4, consistent with the hypothesis that cancers with activated β -catenin overexpress BMP4. Of note, 293 cells, a nontransformed human cell line lacking activated β -catenin, failed to secrete measurable levels of BMP4 protein.

Discussion

This is, to our knowledge, the first demonstration that oncogenic β -catenin can modulate BMP4 expression in human cancer. Regulatory interactions between the Wnt and BMP signaling pathways have been reported in lower eukaryotes; however, whether Wnt signaling induces or represses expression of BMP orthologues has been controversial (12–14). Here, we clearly demonstrate that oncogenic activation of β -catenin signaling leads specifically to overexpression of BMP4 in human cancer.

BMP4 is a member of the TGF- β superfamily of growth factors and was initially identified by its ability to induce bone formation (15). Studies of BMP4 orthologs have revealed that the growth factor has pleiotropic activities in cellular differentiation and organismal development, *e.g.*, classic embryological studies in *Xenopus laevis* embryos have demonstrated that BMP4 is critical for dorsal-ventral patterning of both mesoderm and ectoderm (16). Of note, ventral ectoderm is the precursor to epithelial cells. Additionally, the *Drosophila melanogaster* BMP4 ortholog decapentaplegic plays critical roles in developmental processes, such as formation of the embryonic dorsal-ventral axis and dorsal closure (17).

Members of the TGF- β family of signal transduction molecules are well known to play important roles in cancer pathogenesis. Mutations have been found in two general classes of TGF- β signal transduction molecules: (a) receptors; and (b) intracellular signal transduction molecules known as SMADs (reviewed in Ref. 18). In addition, perhaps most intriguing in light of our finding, it was reported recently that inactivating mutations in the BMP receptor subunit BMPRI1A can cause juvenile polyposis syndrome, a rare gastrointestinal cancer predisposition syndrome (10).

As described above, the vast majority of genetic data suggests that inactivation of signaling by TGF- β family members is a pathogenic mechanism in human cancer. However, the data reported here suggest that activation of BMP4 might be an important mechanism in the pathogenesis of sporadic human colon cancers. We cannot yet reconcile these seemingly disparate observations but expect that they will point us in the direction of underlying fundamental simplification.

In summary, we have identified BMP4 as a gene potentially regulated

by activated β -catenin in human cancer cells. As a molecule well known to be involved in epithelial cell differentiation, BMP4 is a particularly intriguing candidate as a mediator of early events in epithelial cell transformation. Additional work will attempt to identify the biochemical basis for the regulation described herein, correlate BMP4 overexpression with the presence of activated β -catenin in a wide variety of human tumor types, and study the phenotypic consequences of BMP4 overexpression in the pathogenesis of human cancer.

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