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ScienceDirect
Cellular Signalling 19 (2007) 1465–1472
CELLULAR SIGNALLING
www.elsevier.com/locate/cellsig

BMP-induced growth suppression in colon cancer cells is mediated by p21^{WAF1} stabilization and modulated by RAS/ERK

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Received 27 November 2006; received in revised form 15 January 2007; accepted 16 January 2007
Available online 24 January 2007

Abstract

Bone morphogenetic proteins (BMPs) regulate cell differentiation, proliferation, and apoptosis through a canonical SMAD signaling cascade. Absence of BMP signaling causes the formation of intestinal juvenile polyps in the colon cancer-prone syndrome familial juvenile polyposis. As sporadic colon cancers appear to have intact BMP signaling, we evaluated if K-RAS, driving a mitogenic pathway frequently activated in colon cancer, negatively affects BMP growth suppression. We treated non-tumorigenic but activated RAS/ERK FET cells with BMP2, and in combination with pharmacological or genetic inhibition of RAS/ERK, examined BMP-SMAD signaling, transcriptional activity, and cell growth, and also assessed p21^{WAF1} mRNA, transcriptional activation, and protein levels. BMP2 increased nuclear phospho-SMAD1 2-fold, which increased another 2–3 fold when RAS/ERK was inhibited. BMP2 increased BMP-specific SMAD transcriptional activity 2-fold over control and decreased cell growth, but inhibition of RAS/ERK further enhanced BMP-specific transcriptional activity by an additional 1.5–2 fold and enhanced growth suppression by 20%. BMP-induced growth suppression is mediated in part by p21^{WAF1}, not by transcriptional upregulation but by improved p21 protein stability, which is inhibited by RAS/ERK. In colon cancer cells, BMP-SMAD signaling and growth suppression is facilitated by p21^{WAF1} but modulated by oncogenic K-RAS to reduce the growth suppression directed by this pathway.
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Keywords: Bone morphogenetic protein; RAS; ERK; TGFβ; Colorectal cancer

1. Introduction

Bone morphogenetic protein (BMP), a ligand belonging to the TGFβ family, regulates cell growth, apoptosis and differentiation in mesenchymal cells and tissues, but its influence in epithelial-derived cells is not understood. In the autosomal dominantly-transmitted hamartomatous polyposis syndrome, juvenile polyposis (JP), caused by mutations in *BMPRIA* [1] or its downstream effector *SMAD4* [2], affected patients have up to a 12-fold increased lifetime risk for colorectal cancer [3]. Congruent with this, Haramis et.al. found that inhibition of BMP signaling by conditional knockout of *BMPRIA* results in the formation of numerous ectopic intestinal crypts which mimic the histopathology of JP, and which included the frequent occurrence of intraepithelial neoplasia [4]. These investigations suggest a role for intact BMP-SMAD signaling in mediating growth suppression, and repression of *de novo* crypt formation and neoplastic growth. In addition, we have shown that key BMP signaling molecules are present in human colorectal cancers, and that BMP induces growth suppression through the BMPRIA receptor [5]. Thus, BMP appears to suppress normal

Abbreviations: BMP, bone morphogenetic protein; TGFβ, transforming growth factor beta; ERK, extracellular signal-related kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription polymerase chain reaction; DN, dominant negative; JP, juvenile polyposis syndrome.

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doi:10.1016/j.cellsig.2007.01.017

and transformed colon epithelial cells growth, and may be important to prevent colonic neoplastic transformation.

A common finding in colonic neoplasms is activation of RAS and its downstream effectors [6,7]. RAS proteins are proto-oncogenes involved in a variety of signal transduction pathways that normally regulate cellular proliferation, differentiation and death. Activation of RAS (*i.e.* through EGFR stimulation) or oncogenic RAS (*via* mutation and constitutive activation) exerts its effects on multiple downstream effector molecules, one of which is RAF, a serine–threonine kinase that activates MEK1 and 2 kinases, which in turn activate Erk kinases 1 and 2 [8–10]. Once phosphorylated, ERK 1 and 2 are free to phosphorylate other cytoplasmic targets as well as translocate to the nucleus and stimulate the activity of an assortment of transcription factors. Indeed, K-RAS develops constitutively activating mutations at codon 12 or 13 in greater than 50% of colorectal adenomas and cancer, and is considered an early event during the progression of colorectal cancer [11–13].

RAS and its downstream effectors might influence BMP signaling and growth effects. BMPs bind to serine–threonine kinases type I receptors (BMPRIA) and type II receptors (BMPRII) [14] that induce phosphorylation of intracellular SMADs 1, 5, or 8 at their carboxy terminus, at serine 463/465 [15]. The activated SMADs then bind to SMAD4, translocate to the nucleus, and in conjunction with specific DNA factors, activate or repress gene transcription [16]. SMAD1 can also be phosphorylated at its mid-protein linker region by activated Erk kinase (downstream of RAS), slowing or inhibiting nuclear accumulation of BMP-activated SMAD1 in mouse mammary cells [17,18].

Because we have previously shown that BMP signaling appears to be intact in primary colon cancers [5], we evaluated if oncogenic K-RAS, driving a mitogenic pathway frequently activated in colorectal cancer, negatively affects BMP signaling and growth suppression. Negative regulation by activated K-RAS would be a means to inhibit BMPs growth suppression in the absence of mutation of BMP signaling components. Here we utilized FET colorectal cancer cells, considered an early model of colorectal cancer due to alterations in Wnt signaling, RAS, and p53 [19], but are non-tumorigenic in nude mice, to examine any influence that RAS has on BMP signaling. We found that FET cells are growth suppressed in response to BMP2 treatment and that oncogenic K-RAS/ERK activation lessens the response to BMP2 in these cells *via* decreased $p21^{WAF1}$ protein stabilization, a novel mechanism by which BMP exerts its growth suppressive effects.

2. Materials and methods

2.1. Cell culture and treatments

FET cells were maintained in Dulbecco′s Modified Eagle Medium:Nutrient Mix F-12 (D-MEM/F-12) (1X), liquid, 1:1, with L-glutamine and HEPES buffer (Invitrogen Corporation, Carlsbad, CA) containing 10% fetal bovine serum and penicillin G/streptomycin (Invitrogen Corporation, Carlsbad, CA). In some assays, cells were pre-treated with PD98059 (an ERK inhibitor; Calbiochem, San Diego, CA) at a concentration of 5 μM for 30 min prior to 100 ng/ml of BMP2 treatment (R and D Systems, Minneapolis, MN).

2.2. Transfections

Dominant negative K-RAS (DN KRAS; a generous gift from Dr. Rik Derynck, University of California, San Francisco), which inhibits the function of activated RAS, and mock vectors were transiently delivered by Transfectin (Promega, Madison, WI) at a ratio of 3:1 of vector to transfection reagent in OPTI-MEM reduced serum free media (GIBCO Carlsbad, CA). After 2–3 h, IMDM with FBS and penicillin G/streptomycin was added to the transfected cells. Two hours post-transfection, complete media was added, and later the cells were used in the experiments.

2.3. Total cell lysis, and immunoblotting

Cells were lysed using total lysis buffer (12 mM Tris–HCl pH 8.3, 100 mM NaCl, 1% SDS, 1% DCA, 1% Triton X-100, 2 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μM DTT, and 2 mM PMSF). The protein was denatured at 100 °C for 5 min and then loaded onto a 15% polyacrylamide gel. After electrophoresis, the proteins were transferred onto a nylon membrane, blocked for 1 h with 5% milk, and probed overnight with primary antibody at 4 °C. Blotting was done with antibodies to $p21^{WAF1}$ at a 1:200 dilution (OP64 Calbiochem, San Diego, CA), and PTEN at a 1:200 dilution (SC7974, Santa Cruz Biotechnology, Santa Cruz, CA). The following day, several PBS-Tween 0.1% washes were performed along with appropriate secondary antibody incubation. Blotted proteins were detected with horseradish peroxidase-linked secondary antibodies (Sigma, St. Louis, MO) followed by ECL detection (Amersham, Little Chalfont, UK).

2.4. Total RNA extraction and semi-quantitative reverse transcriptase-polymerase chain reaction

Total RNA extraction was performed using Trizol reagent (Invitrogen Corporation, Carlsbad, CA). Cells grown on 6-well plates were lysed with trizol (1 mL/well) and were combined with chloroform and mixed. Supernatants were then precipitated with isopropanol, and the RNA pellets were washed with 75% ethanol and air-dried, and resuspended in water. Two micrograms of total RNA was converted into cDNA by reverse transcriptase and amplified for BMP2 and BMP7 ligand transcripts (SuperScript II, Invitrogen Corporation). Briefly, following inactivation at 65 °C for 10 min, 1 μL of the reaction mixture was incubated in buffer containing 0.2 mM concentrations of dATP, dCTP, dGTP, dTTP, 0.2 μM concentrations each of oligonucleotide primers, 3 mM $MgCl_2$ and a 10X buffer consisting of 200 mM Tris–HCl (pH 8.0), 500 mM KCl, and 1 U Taq polymerase. The following primers were designed to amplify BMP2 and BMP7: BMP2, forward 5′-CCCAGCGTGAAAAGAGAGAC-3′ and reverse 5′GAGACCGCAGTCCGTCTAAG-3′; BMP7, forward 5′-TCGTGGAACAT-GACAAGGAA-3′ and reverse 5′-CTGATCCGGAACGTCTCATT-3′. Primers for p21/Waf1 were as follows: forward 5′CAGGGGACAGCAGAG-GAAGA-3′ and reverse 5′-TTAGGGCTTCCTCTTGGAGAA-3′. Primers for PTEN are forward 5′-GGACGAACTGGTGTAATGATATG-3′ and reverse 5′-TCTACTGTTTTTGTGAAGTACAGC-3′. GAPDH served as a loading control (forward 5′-ACCACAGTCCATGCCATCAC-3′ and reverse 5′-TCCAC-CACCCTGTTGCTGTA-3′). PCR was performed as follows: denaturation at 95 °C for 3 min and optimized amplification for up to 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 74 °C for 4 min for BMP7 and GAPDH; for BMP2: denaturation at 95 °C for 3 min and optimized amplification for up to 40 cycles of 94 °C for 30 s, 57 °C for 30 s, and 74 °C for 4 min; for PTEN: denaturation at 95 °C for 3 min; and for p21: denaturation at 95 °C for 3 min and 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 74 °C for 4 min.

2.5. Luciferase assays

Transient transfection of colon cancer cells with the BRE-Luc plasmid, which contains a consensus promoter element specific to BMP-SMAD activation (a gift from Dr. Peter ten Dijke, Netherlands Cancer Institute, Amsterdam) was done to assess the effects of BMP2 on BMP-specific transactivation. The pWWP-luc plasmid, containing the promoter of $p21^{WAF1}$, was transfected to assess the effects of BMP2 on $p21^{WAF1}$ transactivation, and the PTEN-luc plasmid, containing the wild type protoer of PTEN, was used to assess the effects of BMP2

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