BMP4-Smad Signaling Pathway Mediates Adriamycin-induced Premature Senescence in Lung Cancer Cells

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Cell senescence, an irreversible cell cycle arrest, reflects a safeguard program that limits the capacity of uncontrolled cell proliferation. Treatment of tumor cells with certain chemotherapeutic agents activates premature senescence to decrease the tumorigenicity. Here we show that sublethal concentrations of adriamycin could induce premature senescence in lung cancer cells. Adria mycin treatment resulted in the up-regulation of p16$\text{INK4a}$ and p21$\text{WAF1/cip1}$. We also show that increases of p16$\text{INK4a}$ and p21$\text{WAF1/cip1}$ expression in response to BMP4 were mediated by the Smad signaling pathway. Furthermore, our data revealed that p300 was recruited to cyclin/cyclin-dependent kinase (cdk) promoters p16$\text{INK4a}$ and p21$\text{WAF1/cip1}$. We also show that increases of p16$\text{INK4a}$ and p21$\text{WAF1/cip1}$ expression in response to BMP4 were mediated by the Smad signaling pathway. Furthermore, our data revealed that p300 was recruited to cyclin/cyclin-dependent kinase (cdk) promoters p16$\text{INK4a}$ and p21$\text{WAF1/cip1}$ promoters by Smad1/5/8 to induce the hyperacetylation of histones H3 and H4 at the promoters. The present study provides useful clues to the evaluation of the potentiality of BMP4 as a responsive molecular target for cancer chemotherapy.

Normal cells undergo senescence after a limited number of generations of proliferation (1). Senescent cells remain metabolically active but have lost the ability to undergo further divisions. Generally, somatic cells lose their proliferation potency depending upon the degree of telomere shortening, a process known as the replicative senescence. Recently, however, several groups have reported another type of cellular senescence, termed the premature or rapid senescence, which is provoked by the induced expression of specific genes and is independent of telomere shortening (2). Premature senescence is commonly characterized by a flat enlarged cell morphology, growth arrest, and high acidic β-galactosidase (SA-β-gal)$^3$ activity, and it usually occurs within a week of exposure to sublethal stresses. The ability to induce senescence in a week of time implicates that the telomere of these senescent cells could not be shortened to the threshold length within such a short period of time (3–5).

Many cancer cells exposed to genotoxic stresses undergo permanent cell cycle arrest and acquire a senescence-like phenotype (6). Senescence is a state of permanent growth arrest in which cells are refractory to mitogenic stimuli. Induction of senescence in tumor cells may be a stopgap mechanism in early cancer transitions that are abrogated in the transition to full malignancy (7). Several anticancer chemotherapeutic drugs, such as adriamycin, can cause senescence in solid tumors (8). However, unlike replicative senescence, premature senescence is associated with more rapid kinetics (hence, the term “accelerated senescence”) and telomere dysfunction without overall telomere shortening (9). A number of previous studies showed that breast and lung tumor cells resected from patients who had received neoadjuvant chemotherapy exhibited senescence markers, including positive staining for SA-β-gal, whereas normal surrounding tissues or tumors from untreated patients did not (9, 10). These studies demonstrate the importance of the premature senescence in cancer therapy.

Concomitant with its role in suppressing cancers, cellular senescence is found to be controlled by several tumor suppressor genes including p53 and retinoblastoma in tumor cells (11, 12). Cellular senescence involves the activation of several tumor suppressor proteins and inactivation of several oncogenes via the P53 or RB (retinoblastoma) pathway (13). Furthermore, evidence demonstrating links between cellular senescence and these tumor suppressor pathways has also been obtained. For instance, cells derived from mice in which the gene encoding the p53 or INK4 gene was inactive failed to undergo senescence in response to multiple stimuli, and became cancerous at an early stage (14). Cyclin/cyclin-dependent kinase (cdk) inhibitor P21$\text{WAF1/cip1}$ (hereafter P21), has been discovered to be overexpressed in the process of senescence. Overexpression of P21 led to cell growth arrest, and this process became irreversible and the arrested cells acquired a senescent phenotype (15, 16). P21, which is usually transcriptionally induced by p53-de-
Bone morphogenetic proteins (BMPs) belong to a subgroup of the transforming growth factor-β (TGF-β) superfamily (18). BMPs are phylogenetically conserved proteins, which are essential for embryonic lung development. It has been reported that BMP4 was infrequently expressed in non-small cell lung cancers (19). BMPs elicit their effects through activation of type-1 and type-2 serine/threonine kinase receptors. BMPs and TGF-β/activin receptor-phosphorylated Smads (R-Smads) oligomerize with the common mediator Smad4 (Co-Smad), and after nuclear import, they regulate gene expression by binding to DNA (20). BMP receptors activate Smad1, Smad5, and Smad8, whereas Smad2 and Smad3 are phosphorylated by activin or TGF-β receptors (21–24). Smad6 and Smad7 have been identified as the inhibitory Smads (I-Smads) (25). I-Smads stably interact with activated type-1 receptors and compete with R-Smads for activation by the receptors. Smad6 has also been reported to compete with Co-Smads (Smad4) for complex formation with R-Smads. Moreover, Smad7 inhibits both TGF-β/activin and BMP signaling, and Smad6 efficiently inhibits BMP signaling but only weakly inhibits TGF-β/activin signaling (25–27).

TGF-β1, a member of the TGF-β superfamily, triggered the two distinct cellular senescence programs, i.e. the replicative and premature senescence, in lung cancer cells (3–5). BMP4, another member of the TGF-β superfamily, has been shown to drive A549 cancer cells into replicative senescence in vitro (28); but the mechanisms underlying this process are still unclear. All these data intrigued us to speculate that BMP4 and TGF-β1 might participate in adriamycin-triggered premature senescence in non-small cell lung cancer cells to decrease the tumorigenicity.

In the present study, we identified a novel role of BMP4-Smad signaling in mediating adriamycin-induced premature senescence in lung cancer cells. We showed that overexpression of BMP4 could trigger premature senescence without telomere shortening, in addition to its known action of inducing telomere shortening-dependent replicative senescence in vitro (28). Moreover, we identified that cell cycle regulatory proteins p16 and p21 were required for BMP4 induced-premature senescence, in which Smad signaling molecules and coactivator p300 played a role. Based on the experimental data generated from this study, we demonstrate the molecular interactions and signaling pathways in adriamycin-induced senescence and identify the role of BMP4 in the control of certain lung cancers.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The P16\(^{INK4a}\) (hereafter P16) promoter reporter (~869 to +1 bp from the cap site) ligated to the luciferase reporter gene (pGL2 basic, Promega) was provided by Dr. E. Hara (Imperial Cancer Research Fund Laboratories, London). The pcDNA3-myc-Smad6 expression plasmid was provided by Dr. S. Itoh (Department of Experimental Pathology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Japan). The plasmid of the p21-luc (2400/+11) was a gift from Dr. Bert Vogelstein (The Johns Hopkins University School of Medicine, Baltimore, MD). The pSTAR vector was kindly provided by Dr. Q. Zeng, (Institute of Molecular and Cell Biology, Singapore). The pSTAR was constructed with neomycin resistance for selection of stably transfected cells, and a cloning cassette for placing a gene of interest under the control of the tetO DNA motif. This plasmid expresses rtTAnls, which upon association with tetracycline, binds to and drives gene expression from the tetO DNA motif. The pEGFP-hBMP4 expression plasmid was provided by Dr. X. Q. Jiang (The Ninth People’s Hospital, Shanghai Second Medical University). hBMP4 cDNA was amplified from pEGFP-hBMP4 plasmid. A 1.2-kb DNA fragment containing the entire coding region of BMP4 was ligated to pSTAR. Recombinant plasmids with the 1.2-kb fragment inserted in the correct orientation were characterized by restriction enzyme digestion. The purified pSTAR/hBMP4 was used for transfection.

**Cell Culture, Transfection, and Luciferase Reporter Assay—**NCI-H460, HCT116, and A549 cells were maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum, 100 mg/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37 °C. Transient transfection of A549, HCT116, and NCI-H460 cells were performed using the Lipofectamine 2000 (Invitrogen) and the FuGENE HD transfection (Roche) procedures, respectively. Transfected cells were assayed for luciferase reporter activity using a Promega dual-luciferase reporter assay system, and the Renilla luciferase control plasmid pREP7-RLuc was co-transfected for normalization.

**Construction of Stably Transfected Cell Lines—**NCI-H460 cells were transfected with pSTAR/hBMP4 or pSTAR using the FuGENE HD transfection reagent (Roche). Cells were maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum, 100 mg/ml penicillin, 100 mg/ml streptomycin, and G418 (1000 μg/ml). After 15–22 days, individual colonies were isolated and expanded. Stably transfected cell lines were maintained in IMDM supplemented with 10% fetal bovine serum in the presence of G418 (1000 μg/ml). The cell line that inducibly expresses BMP4 protein was designated the pSTAR-hBMP4. Induction of BMP4 expression by doxycycline (Clontech) was both dose- and time-dependent in the pSTAR-hBMP4 cell line. The cell line transfected with pPTEt-on only, designated the pSTAR, was used as a control.

**RNA Extraction, Reverse Transcription-PCR (RT-PCR), and Real-time PCR Analyses—**Total cellular RNA was extracted from A549, HCT116, and NCI-H460 cells according to the Promega Total RNA Isolation System manual. RT-PCR was performed using the Access RT-PCR System supplied by Promega. Real-time PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems), following the manufacturer’s protocol, with SYBR Green (TaKaRa, Osaka, Japan) used as a double-stranded DNA-specific fluorescent dye. The primer pairs for the P16 gene were: sense, 5’-tctctggacagcgt-gtt-3’ and antisense, 5’-caacggaggagttc-3’. The primer pairs for the P21 gene were: sense, 5’-gggtgctcgtgagacc-3’ and antisense, 5’-gctgctccgctgga-3’. The primer pairs for BMP4 gene were: sense, 5’-agcgtcagattaacg-3’ and antisense, 5’-tgagtgaggcagagtc-3’. TGF-β1 gene were: sense,
5′-ccaactattgcttcagctcca-3′ and antisense, 5′-ttatgctggttgtacaggg-3′. The β-actin primer pairs were: sense, 5′-tcgtgcgtgacattaaggag-3′ and antisense, 5′-atgccagggtacatggtggt-3′.

Western Blotting—Cells were harvested after treatments and lysed in the lysis buffer for 30 min at 4 °C. Total cell extracts were separated in 12% SDS-PAGE, and then transferred to a polyvinylidene fluoride membrane. The membrane was incubated with anti-p16 (Santa Cruz), anti-myc (Upstate Biotechnology, NY), anti-BMP4 (Santa Cruz), anti-p53 (Abcam), anti-p21 (Santa Cruz), anti-Phospho-Smad1 (Ser-463/465)/Smad5(Ser-463/465)/Smad8(Ser-426/428) (Cell Signaling Technology), or anti-β-actin (Sigma) antibodies. The signals were visualized by using the Chemiluminescent Substrate method with the SuperSignal West Pico kit provided by Pierce Co.

Cellular Immunofluorescence—Cells were fixed with 1% formaldehyde in culture medium for 10 min at 37 °C and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 10 min at 4 °C. Trimethylated histone H3 was detected with antibody against tri-Me-K9H3 antibody (Abcam) and visualized with a fluorescein isothiocyanate-conjugated anti-rabbit IgG secondary antibody (Zhongshan, China). Photographs were taken with a confocal microscopy.

Chromatin Immunoprecipitation (ChIP)—ChIP assays were carried out using a kit supplied by Upstate following the manufacturer’s protocol. Cells were cross-linked with 2% formaldehyde for 10 min at 37 °C, and then lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1) with protease inhibitors. The sonicated lysates were processed using a ChIP assay kit, essentially as described by the manufacturer (Upstate). DNA was immunoprecipitated with antibodies against Ac-H3 (Upstate), Ac-H4 (Upstate), p300 (Santa Cruz), and phospho-Smad1/5/8. The precipitated chromatin was analyzed by quantitative real-time PCR using SYBR Green.
BMP4 Mediates Adriamycin-induced Premature Senescence

**FIGURE 2.** BMP4 was required for the premature senescence induced by adriamycin in lung cancer cells. A, Western blotting confirmation of siRNA-mediated BMP4 knockdown. The negative control was an irrelevant siRNA. B, knockdown of BMP4 resumed the proliferation of NCI-H460 cells and A549 cells inhibited by adriamycin. Cells transfected with BMP4 siRNA vectors were treated with adriamycin for 3 days and cell proliferation was tested by MTT assay. *, p < 0.05; **, p < 0.01 versus untreated group. #, p < 0.05; ##, p < 0.01 versus adriamycin-treated group. Data are based on three independent experiments. C–E, BMP4 restrained cell senescence induced by adriamycin. NCI-H460 and A549 cells were transfected with BMP4 siRNA, and then treated with adriamycin at 500 nM and 2 μM, respectively. The senescence was examined by SA-β-gal activity assay, SA-β-gal staining, and immunofluorescence. *, p < 0.05; **, p < 0.01 versus untreated group. #, p < 0.05; ##, p < 0.01 versus adriamycin-treated group. Data are based on three independent experiments. F, Western blotting confirmation of BMP4 overexpression in the pSTAR-hBMP4 stably transfected cell line. pSTAR-hBMP4 cells were treated with DOX for 24 h, and Western blot was carried out with antibody against BMP4. pSTAR cells treated with DOX was used as the control. G, Western blotting detection of BMP4 overexpression in pSTAR-hBMP4 cells treated with 10 μg/ml DOX for the indicated days. H, Western blotting analysis of the mount of BMP4 overexpression in pSTAR-hBMP4 cells treated with 10 μg/ml DOX, in comparison with that in NCI-H460 cells treated with adriamycin. I, BMP4 inhibited the proliferation of NCI-H460 cells by MTT assay. pSTAR (left panel) and pSTAR-hBMP4 (right panel) cells were seeded on 96-well plates at a density of 2 × 10^3 cells/well, respectively. After treatments with DOX for the times indicated, cell viability was assessed by measuring absorbance at 492 nm with a microplate reader. J and K, BMP4 induced premature senescence in NCI-H460 cells. SA-β-gal activity assay and SA-β-gal staining was performed on cells treated with DOX for 6 days. NCI-H460 cells treated with adriamycin were used as a positive control. *, p < 0.05; **, p < 0.01 (n = 3). L and M, BMP4 overexpression led to the appearance of heterochromatic DNA foci.

dye. The primers specific to sequences at the P16 promoter were: P1 sense, 5'-cattgcgaattgctggagt-3', antisense, 5'-ctggctctccgcgcgcggctgccgtc-3'; P2 sense, 5'-tagaagggttgtatggcggaggg-3', antisense, 5'-caagggagggagtgcgcctc-3'; and P3 sense, 5'-agacacggcttcttacaagcag-3', antisense, 5'-ccaggagaatggaatcacc-3'. The primers specific to sequences at the P21 promoter were: Q1 sense, 5'-aaagcgacagctgttgaagtg-3' (29) and 5'-aaagcgacagctgttgcaagga-3' (30), respectively. The BMP4-specific siRNA sequence was 5'-gagggacacgctttacagt-3' (31). The P53-specific siRNA sequence was 5'-gaatgcgcgttgaatcctc-3' (32). The control siRNA sequence was 5'-ctggcaactatgtgccctg-3'. Oligonucleotides that represent small hairpin RNAs targeting these sequences were designed, synthesized, and cloned into the pSilencer4.1-CMV neo vector (Ambion) between BamHI and HindIII sites according to the manufacturer's instructions.

Cell Proliferation and MTT Assay—Cell proliferation was measured by using the MTT assay. After transfection or treatments, cells were incubated with 5 mg/ml MTT solution for 4 h. The medium was aspirated, and the formazan product was solubilized with 100 μl of dimethyl sulfoxide. Viability was assessed by measuring the absorbance at 492 nm with a microplate reader.

Senescence-associated Galactosidase (SA-β-gal) Activity Assay and Cytochemical Staining for SA-β-Galactosidase—Cells were lysed in reporter lysis buffer. Cell lysates containing equal amounts of total protein were diluted in equal volumes of 2× assay buffer containing 1.33 mg/ml o-nitrophenyl-d-galactopyranoside, 2 mM MgCl₂, and 100 μl of 2-mercaptoethanol in 200 mM phosphate buffer (pH 6.0), and incubated at 37 °C for
4 h. The absorbance at 420 nm was measured after addition of an equal volume of 1 M Na₂CO₃.

Cytochemical staining for SA-β-galactosidase was performed using a Senescence β-Galactosidase Staining Kit (Cell Signaling Technology) at pH 6.0. All the experiments were repeated three times, and one of the representative results is shown.

**RESULTS**

Adriamycin-induced Premature Senescence in Company with the Up-regulation of BMP4 in Lung Cancer Cells—Because growth arrest is a necessary step leading to cell senescence, we first determined whether adriamycin treatment affected cell proliferation. MTT assays showed that adriamycin was able to decrease the proliferation indices of NCI-H460 and A549 cells at 500 nM and 2 μM, respectively (Fig. 1A). We then found that NCI-H460 and A549 cells treated with adriamycin exhibited phenotypic changes that resembled those observed in cells undergoing senescence, including the intensified SA-β-gal staining, flattened cell morphology, and enlarged cell size, in just 1 week (Fig. 1B). We next examined the endogenous SA-β-gal (pH 6.0) activity. As shown in Fig. 1C, a great increase in SA-β-gal activity was seen 6 days after adriamycin treatment, in comparison with untreated cells. Because the occurrence of heterochromatin foci is a typical marker in human cells undergoing senescence; we therefore examined whether adriamycin was able to induce heterochromatin foci. As shown in Fig. 1D, the heterochromatin foci were readily visible in senescent cells treated with adriamycin by 4',6-diamidino-2-phenylindole staining. Concurrently, trimethylated histone H3 at lysine 9 (3meK9H3), a well characterized heterochromatin mark (33), was co-localized with specific heterochromatic foci. The percentage of cells containing heterochromatic foci was greatly increased when the tumor cells were treated with adriamycin (Fig. 1E). These results indicated that adriamycin could induce tumor cell senescence to impair the tumorigenicity in non-small cell lung cancer cells. Furthermore, NCI-H460 cells were more sensitive to adriamycin than A549 cells. TGF-β1 and BMP4, two members of the TGF-β superfamily, have been reported to induce lung cancer cells into replicative
senescence (3, 28). We then asked whether BMP4 and TGF-β1 were associated with the cellular senescence induced by adriamycin. As shown in Fig. 1, F and G, RT-PCR and quantitative real-time PCR data demonstrated that the BMP4 mRNA level was greatly up-regulated when NCI-H460 and A549 cells were treated with adriamycin at 500 nM and 2 μM, respectively, whereas TGF-β1 expression was not markedly changed. Meanwhile, the BMP4 protein level was also significantly elevated by adriamycin (Fig. 1H). These results indicated that BMP4 may participate in the adriamycin-induced premature senescence in lung cancer cells.

BMP4 Played a Key Role in Mediating Adriamycin-induced Senescence in Lung Cancer Cells—To further characterize the function of BMP4 in adriamycin-induced senescence, we used the RNAi approach to knockdown BMP4 expression to assess its effects. The endogenous BMP4 protein expression was markedly reduced by transfection of specific BMP4 siRNA as confirmed by Western blotting (Fig. 2A). We then showed that suppression of endogenous BMP4 expression restrained the adriamycin-induced senescence (Fig. 2, B–E), eliciting the critical role of BMP4 in this process. To study how BMP4 mediated adriamycin-induced premature senescence, we constructed a stably transfected cell line that can be induced to express BMP4 protein by doxycycline (DOX). Western blotting was performed to verify the DOX inducibility of the transgenes in the pSTAR-hBMP4 cell line carrying the full-length BMP4 construct. Representative results are presented in Fig. 2, F and G, which show that induction of the BMP4 expression by DOX was both dose- and time-dependent. Moreover, Western blots revealed that the induction of BMP4 expression by DOX was well equivalent to the extent of BMP4 up-regulation by adriamycin (Fig. 2H), implying that induction of BMP4 expression by DOX in the pSTAR-hBMP4 cell line was able to imitate the biological process of premature senescence initiated by up-regulation of BMP4 in response to adriamycin. As shown in Fig. 2I, right, DOX-stimulated BMP4 expression decreased the proliferation index of NCI-H460 cells in a dose-dependent manner, and the proliferation ability of NCI-H460 cells was decreased greatly at 48 h as judged by MTT assays (Fig. 2I, right). In contrast, DOX treatment of pSTAR cells did not undergo changes in proliferation index, suggesting that DOX itself had no inhibitory effect on cell proliferation (Fig. 2I, left). Meanwhile, pSTAR-hBMP4 cells were triggered into senescence when they were treated with

FIGURE 3. Senescence triggered by BMP4 was dependent upon the Smad signaling pathway. A, Western blotting confirmation of the ectopic expression of Smad6 in NCI-H460 cells. B, Smad6 restrained cellular senescence induced by BMP4. pSTAR-hBMP4 cells transfected with Smad6-myc expression plasmid were exposed to DOX for 6 days and tested for the SA-β-gal activity. The pcDNA3.1 empty vector was used as a negative control. *, p < 0.05 versus untreated group. #, p < 0.05, versus the DOX-treated group. Data are based on three independent experiments. C, representative photomicrographs of the SA-β-gal staining. D, the average percentages of cells containing heterochromatin foci were greatly decreased upon Smad6 overexpression. E–G, Smad6 repressed cellular senescence induced by adriamycin as judged by SA-β-gal activity assays, SA-β-gal staining, and immunofluorescence. *, p < 0.05; **, p < 0.01 versus untreated group. #, p < 0.05; ##, p < 0.01 versus adriamycin-treated group. Data are based on three independent experiments. H, BMP4 elevated the phosphorylation levels of the downstream proteins Smad1/5/8. Western blotting estimation of the phosphorylation levels of Smad1/5/8 in pSTAR-hBMP4 cells treated with DOX.
DOX for 6 days (Fig. 2, J–M). These results indicated that hBMP4 overexpression was able to induce the onset of senescence in lung cancer cells in 1 week. Numerous studies have highlighted that the telomere of the senescent cells would not shorten to the threshold length in 1 week (3–5). Therefore, the above data may hint at a new role of BMP4 in triggering premature senescence in cancer cells, in addition to its reported role in causing replicative senescence in A549 cells.

The Adriamycin-induced Senescence Was Dependent upon the BMP-Smad Signaling Pathway—Next, we sought to determine the intracellular signaling pathway initiated by BMP4 in mediating adriamycin-triggered senescence. Because the BMP-Smad pathway was reported to be inhibited by Smad6 (25, 27), we wanted to know whether BMP4-induced senescence was abrogated when the Smad pathway was interrupted by Smad6 overexpression. The ectopic expression of Smad6-myc was first confirmed by Western blot analysis (Fig. 3A). We then showed that suppression of the BMP-Smad pathway by Smad6 did interfere with the BMP4-induced premature senescence, as revealed by SA-β-gal activity assay, SA-β-gal staining, and cellular immunofluorescence (Fig. 3, B–D). Furthermore, Smad6 overexpression nearly abrogated the senescence triggered by adriamycin (Fig. 3, E–G). We then examined whether BMP4 was able to activate the BMP-Smad signaling pathway by enhancing phosphorylation levels of downstream proteins Smad1/5/8. As shown in Fig. 3H, the phosphorylation levels of Smad1/5/8 were significantly increased in pSTAR-hBMP4 cells treated with DOX. Together, our results suggested that the Smad pathway activated by BMP4 played an important role in modulating adriamycin-induced premature senescence.

The Premature Senescence Mediated by the BMP4-Smad Pathway Required the Participation of p16

FIGURE 4. p16 and p21 were required for BMP4-induced premature senescence. A. BMP4 overexpression led to increases in p16 and p21 protein levels. Lysates were prepared 2 days following DOX treatment and probed with the indicated antibodies. β-Actin was used as an internal reference control. B, knockdown of BMP4 restrained the increased expression of p16 and p21 in response to adriamycin. NCI-H460 cells transfected with the BMP4 siRNA plasmid were treated with adriamycin, and then subjected to Western analysis with the p21 or p16 antibodies. C, Smad6 repressed BMP4-stimulated p16 and p21 expression. pSTAR-hBMP4 cells transfected with the Smad6-myc expression plasmid were treated with DOX, and then subjected to Western analysis with the p21 or p16 antibodies. D, Western blotting confirmation of p16 and p21 knockdown with p21 siRNA or p16 siRNA. The negative control was an irrelevant siRNA. E, knockdown of p16 and p21 resumed the proliferation of NCI-H460 cells inhibited by BMP4. pSTAR-hBMP4 cells transfected with p16 siRNA, p21 siRNA, or p16 siRNA plus p21 siRNA vectors were treated with DOX and cell proliferation was tested by MTT assay. *, p < 0.05; **, p < 0.01 versus untreated group. #, p < 0.05; ##, p < 0.01 versus DOX-treated group. Data are based on three independent experiments. F, knockdown of p16 and p21 inhibited premature senescence of NCI-H460 cells induced by BMP4. pSTAR-hBMP4 cells transfected with p16 siRNA, p21 siRNA, or p16 siRNA plus p21 siRNA vectors were treated with DOX and tested by SA-β-gal staining. G, BMP4 enhanced the endogenous SA-β-gal activity. An irrelevant control siRNA vector was used as the control. *, p < 0.05; **, p < 0.01 versus untreated group. #, p < 0.05; ##, p < 0.01 versus DOX. Data are based on three independent experiments. H, knockdown of p16 and p21 inhibited the formation of heterochromatin foci induced by BMP4. The average percentage was calculated based on three independent fields. *, p < 0.05; **, p < 0.01 versus untreated group. #, p < 0.05; ##, p < 0.01 versus DOX-treated group. Data are based on three independent experiments.
and p21—Cell cycle regulatory proteins p53, p21, and p16 were reported to play roles in cell senescence (11, 12, 15). We then speculated that the BMP4-mediated premature senescence might involve changes in expression of these regulators. Our Western blotting analysis confirmed that BMP4 overexpression stimulated p16 and p21 expression; however, there was little change in expression of the p53 protein (Fig. 4A). Furthermore, increases of p16 and p21 expression in response to adriamycin were remarkably attenuated when BMP4 expression was knocked down by the RNA interference strategy (Fig. 4B). In addition, we found that the increases of p16 and p21 expression in response to BMP4 were remarkably attenuated in SMAD6 transfectants (Fig. 4C). We next asked whether p16 and p21 proteins were required for BMP4-mediated senescence. To test this, we employed the RNAi method to knockdown p21 or p16 expression in pSTAR-hBMP4 cells. Western blotting results confirmed that the endogenous p21 and p16 expression levels were decreased by their respective siRNAs (Fig. 4D). We then demonstrated that the capacity of DOX on reduction of proliferation of pSTAR-hBMP4 tumor cells could be effectively restored by knockdown of endogenous p16 and p21 (Fig. 4E). Furthermore, suppression of endogenous p21 and p16 restrained the senescence induced by BMP4 (Fig. 4, F–H). These results provided evidence that p16 and p21 were required for BMP4-mediated premature senescence.

BMP4 Mediates Adriamycin-induced Premature Senescence

BMP4 induced transcription of p16 and p21 by increasing Smad1/5/8 transcriptional activity. A, BMP4 up-regulated the endogenous P16 and P21 mRNA levels in NCI-H460 cells. pSTAR-hBMP4 cells were treated with DOX for 2 days, and P16 or P21 mRNA was measured by RT-PCR. B, quantitative estimation of P16 and P21 mRNA using real-time PCR. C, BMP4 up-regulated P16 and P21 promoter activities. 1 μg of the pEGFP-hBMP4 expression plasmid, together with 1 μg of P16 or P21 reporter plasmid were cotransfected into NCI-H460 cells. Luciferase activity was determined 30 h after transfection and normalized to the Renilla activity. The pcdNA3.1 plasmid was used as a negative control. *, p < 0.05; **, p < 0.01 (n = 3). D, diagrams of the 5' flanking regions of P16 and P21 genes. Lines below (P1–P3) and (Q1–Q3) indicate the three regions of the P16 and P21 promoter amplified in ChIP assays, respectively. E, BMP4 overexpression potentiated the binding of phospho-Smad1/5/8 at different regions of P16 and P21 promoters, as revealed by ChIP assays. pSTAR-hBMP4 cells were treated with DOX for 2 days and harvested for ChIP assay. Samples were immunoprecipitated with anti-phospho-Smad1/5/8 antibodies. Precipitated DNAs were amplified by using quantitative PCR.

Our data also showed that BMP4 up-regulated the P21 and P16 promoter activities by 3- and 4-fold, respectively, as determined by luciferase reporter assays (Fig. 5C). These data, together with the above findings of the effect of BMP4 on increasing phosphorylation levels of Smad1/5/8 (Fig. 3H), had prompted us to speculate that transcription factors Smad1/5/8 might participate in BMP4-mediated up-regulation
Histone Acetylation Played an Important Role in Transcriptional Regulation of p16 and p21—Our previous studies showed that histone acetylation modifications were involved in transcriptional regulation of p16 and p21 (34, 35). We were then interested in finding out whether this epigenetic mechanism was also effective in the process of BMP4-stimulated p16 and p21 up-regulation. To do this, ChIP assays were performed to estimate histone acetylation levels at the P16 and P21 promoters using antibodies against Ac-H3 and Ac-H4. The results validated that BMP4 overexpression increased the acetylation levels of histones H3 and H4 at the Smad-binding domains of the P16 and P21 promoters (Fig. 6, A and B, and supplemental Fig. S3A).

Histone acetyltransferase p300 has been reported to play an important role in ras-induced premature senescence (36). We then intended to determine whether p300 participated in BMP4-mediated premature senescence. ChIP assays were performed to detect the possible presence of the p300 protein at P16 and P21 promoters in the process of BMP4-mediated premature senescence, and the results showed that overexpression of BMP4 increased the enrichment of p300 at the P16 promoter, especially at the P1 and P2 regions (Fig. 6C, and supplemental Fig. S3B, left). Similarly, p300 protein was also enriched at the Q2 and Q3 regions of the P21 promoter, but not at the Q1 region (Fig. 6C, and supplemental Fig. S3B, right). These data suggested that the enrichment of p300 at P16 and P21 promoters may account for the increased acetylation levels of H4 and H3 at the same promoter regions (Fig. 6, A and B, and supplemental Fig. S3A).

p300 Was Recruited to P16 and P21 Promoters by Smad1/5/8—To determine whether p300 was recruited to P16 and P21 promoters by Smad1/5/8, we tested the presence of p300 at different promoter regions of P16 and P21 when the binding abilities of Smad1/5/8 to these regions were decreased by Smad6 overexpression. As shown in Fig. 7A and supplemental Fig. S4A, the binding abilities of Smad1/5/8 to the P16 and P21 promoters were significantly decreased in pSTAR-hBMP4 cells transfected with the Smad6 expression plasmid, which is known to inhibit BR-Smad phosphorylation and nuclear translocation (25, 27). Meanwhile, the enrichment of p300 at P16 and P21
**BMP4 Mediates Adriamycin-induced Premature Senescence**

![Diagram of p300 recruitment to P16 and P21 promoters by transcription factors Smad1/5/8](image)

A, the binding abilities of Smad1/5/8 were declined upon Smad6 overexpression. pSTAR-hBMP4 cells were transfected with Smad6 expression plasmid and treated with DOX before being subjected to ChIP assays. Samples were immunoprecipitated (IP) with anti-phospho-Smad1/5/8 antibodies and amplified by quantitative PCR. B, the enrichments of p300 at different regions of P16 and P21 promoters were significantly decreased when the binding abilities of Smad1/5/8 were restrained by Smad6 overexpression. ChIP assays were performed with antibody against p300. C and D, ChIP assays for detection of the presence of acetylated histones H3 and H4 at p16 and p21 promoters in pSTAR-hBMP4 cells transfected with Smad6 expression plasmid. E, H3 acetylation level of p16 promoter. F, H4 acetylation level of p21 promoter.

**DISCUSSION**

Senescence in tumors is considered a major criterion for effective cancer therapies, and it plays a pivotal role in safeguarding organisms against tumorigenesis by suppressing the emergence of immortal cells (37). Identification of molecules responsible for controlling premature senescence may facilitate the development of new cancer treatment modalities. Our data in this report validated that adriamycin was an effective chemotherapeutic drug functioning through triggering premature senescence in lung cancer cells (Fig. 1, A–E). Moreover, we revealed that overexpression of BMP4 played a critical role in mediating adriamycin-induced senescence. Suppression of endogenous BMP4 expression restrained the adriamycin-induced senescence in lung cancer cells (Fig. 2, B–E). Meanwhile, we found that BMP4 also participated in mediating adriamycin-triggered senescence in colon cancer cells (supplemental Fig. S5, A–K). These data implicated that BMP4 may be a new molecular target for the chemotherapeutic drug adriamycin. Although BMP4 has been reported to cause telomere shortening-dependent replicative senescence in vitro (28), our study suggested that overexpression of BMP4 was also able to trigger premature senescence (Fig. 2, I–M). These data provided useful information for the exploration of a feasible strategy for cancer treatment based on induction of premature senescence.
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Premature senescence has been reported to be often provoked by the accumulation of tumor suppressors such as p53, p21, and p16 (11, 12, 15). Data presented in this report demonstrated that the adriamycin-induced senescence was associated with the increase of p53 expression (supplemental Fig. S6A), which is consistent with the results from Elmore et al. (8). However, we detected that the adriamycin-induced senescence was only partially restrained by knockdown of p53 expression using an RNAi strategy in lung cancer cells (supplemental Fig. 6, C–F), implying that some other tumor suppressors may also participate in mediating adriamycin-induced senescence. We were then curious to find out which tumor suppressors participate in this process. BMP4 and TGF-β1 were the first candidates we were interested in because they played key roles in inducing senescence in lung cancer cells (3, 28). Our results demonstrated that the adriamycin-induced senescence was accompanied by an up-regulation of BMP4 expression (Fig. 1, F and G). Moreover, knockdown of BMP4 expression by RNAi restrained the senescence induced by adriamycin (Fig. 2, B–E). Meanwhile, significant increases in expression of the senescent-associated tumor suppressors such as p21 and p16 in response to adriamycin were restrained by BMP4 knockdown (Fig. 4B). These results indicated that BMP4 played an important role in modulating adriamycin-induced premature senescence. In addition, BMP4-induced senescence was associated with significant increases in p21 and p16 expression (Fig. 4A); but we did not observe any difference in the expression level of the cell cycle regulatory protein p53 during the process of BMP4-induced senescence (Fig. 4A), implicating that the induction of p21 by BMP4 may be independent of p53 accumulation. The induction of cell senescence by p21 in a p53-independent fashion was also reported by Trost et al. (38). Moreover, our results showed that the adriamycin-induced senescence was nearly abrogated by knockdown of both BMP4 and p53 using RNAi (supplemental Fig. S7, A–D). Thus, data presented in this report demonstrated that both BMP4 and p53 may play important and independent roles in mediating adriamycin-induced premature senescence.

BMPs, members of the TGF-β superfamily, exert diverse functions in many biological contexts. For example, they play roles in limb development, generation of primordial germ cells, tooth development, cell apoptosis, differentiation, etc. (39). In the present study, we identified a novel role of BMP4-Smad signaling in mediating adriamycin-induced premature senescence (Fig. 3, B–G, and supplemental Fig. S8, A–D). We showed that suppression of the BMP-Smad pathway by Smad6 did interfere with the BMP4-induced premature senescence (Fig. 3, B and C). It has been shown that P21 expression is regulated by transcription factor Smad1 (40–42). We established in this study that p21 was also up-regulated through Smad pathway (supplemental Fig. S1, A and B, and Fig. 5E). Significantly, we discovered that P16 was a new responsive gene of BMP4 action, and this process was also controlled by the Smad pathway (supplemental Fig. S1, A and B, and Fig. 5E). Furthermore, we found that BMP4 could elevate the phosphorylation levels of the downstream proteins Smad1/5/8 to active Smad signaling pathway (Fig. 3H). These studies provide evidence that the Smad pathway activated by BMP4 plays a crucial role in BMP4-induced premature senescence. However, the functional difference between these BR-Smads was not investigated in this study. Further studies will be needed to identify which of these BR-Smads plays the most crucial role in inducing premature senescence by BMP4.

A significant point arising from this investigation has been the finding that histone acetylase p300 played a role in mediating senescence through acetylating the histones at the promoter regions of P16 and P21 (Fig. 6, A and B, and supplemental Fig. S3A). The identification of the role of p300 in senescence is consistent with the results reported by Deng et al. (36). Moreover, our results demonstrated that p300 was recruited to P16 and P21 promoters by Smad1/5/8 in BMP4-induced senescence (Fig. 7B and supplemental Fig. S4B). The interaction of p300 with transcription factors, such as BR-Smads/Smad4, may facilitate its recruitment to target promoters and to acetylate histones through its acetyltransferase activity, thereby loosening the chromatin structure and increasing the accessibility of the preinitiation complex to RNA. This has implicated the potential function of p300 in mediating cellular senescence responses for the tumor suppressing.

To conclude, we established in this study, for the first time, that up-regulation of BMP4 induced by adriamycin played a critical role in mediating adriamycin-triggered premature senescence in lung cancer cells. We also demonstrated that the Smad signaling pathways activated by BMP4 was an important pathway in modulating adriamycin-induced cell senescence. Moreover, overexpression of BMP4 alone was able to cause the premature senescence in lung cancer cells, and this process required the elevation of p16 and p21 expression. These data hint that BMP4 may be effective in the control of certain lung cancers and it may be a key gene in response to chemotherapeutic drugs.

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