Targeted Repression of Bone Morphogenetic Protein 7, a Novel Target of the p53 Family, Triggers Proliferative Defect in p53-Deficient Breast Cancer Cells

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Abstract

p53 tumor suppressor and its family members, p63 and p73, are known to play a role in the survival of cells exposed to stress signals. As a transcription factor, the p53 family proteins induce a plethora of target genes that mediate their functions in the cell cycle, apoptosis, and other biological activities. However, the mechanism by which the p53 family proteins regulate their cell survival functions is still not clear. Here, we showed that bone morphogenetic protein 7 (BMP7) is a novel target gene regulated by the p53 family and mediates the cell survival function of the basal physiologically relevant level of p53. Specifically, we found that knockdown of BMP7 markedly inhibits the proliferation of p53-deficient, but not p21-knockdown, breast cancer cells compared with the ones with wild-type p53. In addition, we found that inhibitor of differentiation or DNA binding 2 (Id2), a transcription factor implicated for cell survival, is regulated by the BMP7 and p53 pathways. Interestingly, whereas a functional BMP7 or p53 pathway is sufficient to maintain the basal level of Id2 expression, loss of both pathways abrogates Id2 expression. Furthermore, we showed that overexpression of Id2 can restore p53-deficient cells to survive in the absence of BMP7. As a result, we identified a previously unrecognized role for BMP7 in the maintenance of cell survival for p53-deficient cells, at least in part, through Id2. Together, we hypothesize that breast cancer patients with mutant p53 might benefit from targeted repression of BMP7 expression and/or targeted inhibition of the BMP7 pathway. [Cancer Res 2007;67(19):9117–24]

Introduction

Bone morphogenetic protein 7 (BMP7), a secreted signaling polypeptide, is a member of the BMP subfamily of the transforming growth factor-β superfamily. Numerous observations over the years have defined BMP7 as a crucial regulator of early embryogenesis and subsequent organogenesis and tissue homeostasis (1, 2). Like the other members of the BMP subfamily, BMP7 signaling is elicited through specific serine/threonine kinase receptors and their downstream effectors, known as Smad1, Smad5, and Smad8 (3–5). On ligand stimulation, these receptor-regulated Smads become phosphorylated by activated type I receptor kinases and form heteromeric complexes with the common mediator Smad4. Finally, activated Smad proteins translocate into the nucleus where they bind to DNA and directly regulate gene expression.

BMP7, originally identified as osteogenic protein 1, has an activity in regulating multiple cellular processes including cell proliferation, migration, and apoptosis. Interestingly, BMP7 is found to have two opposing effects on cell proliferation, dependent on the type and differentiation state of the cells (6, 7). For example, in prostate and anaplastic thyroid carcinoma cells, BMP7 inhibits cell proliferation by arresting cells in G1 phase via up-regulation of the cyclin-dependent kinase (Cdk) inhibitors, p21 and p27, and decrease in the activity of Cdk2 and Cdk6 (8, 9). However, in C4-2B cells, BMP7 promotes cell survival by inhibiting stress-induced apoptosis via both the Smad/survivin and c-jun NH₂-terminal kinase pathways (10).

The BMP7 gene is frequently found to be amplified and overexpressed in breast cancer cell lines and tumor samples. One study showed that overexpression of BMP7 occurs in up to 71.4% of primary breast cancers and is associated with a high histologic grade of tumors (11). Consistent with this, ablation of the type II BMP receptors in breast cancer cells leads to growth inhibition, suggesting that BMPs have a growth-promoting function in breast cancer cells (12). However, the molecular mechanism underlying the role of BMP7 in breast cancers is still poorly understood.

p53 is the most frequently mutated gene in human cancers including breast cancer. Mutation of p53 abrogates p53 DNA binding and transcriptional activities. As a transcription factor, p53 transactivates its downstream target genes by binding to p53 responsive elements (p53-RE) in the promoter or intron, such as p21 (WAF1/CIP1; ref. 13), murine double minute 2 (14), insulin-like growth factor binding protein 3 (15), and Killer/death receptor 5 (16). These target genes mediate diverse biological functions of p53, including cell cycle arrest and apoptosis (17). In addition, recent accumulating evidence indicates that physiologically relevant low levels of p53 serve as a cell survival factor (18–21). However, the mechanism responsible for p53-dependent cell survival remains to be elucidated.

As members of the p53 family, both p63 and p73 can bind to the canonical p53-RE, transactivate p53 target gene expression, and induce biological functions similar to that for p53 when overexpressed or activated. Interestingly, unlike p53, the genes encoding p63 and p73 are rarely mutated in human cancers, and neither of the p63 and p73 knockout mice exhibits a propensity for tumor formation. These animals show rather discrete developmental defects (22, 23). Thus, whereas p53 is important for the prevention of cancer, both p63 and p73 are crucial for normal development.

Here, we report that BMP7 is a novel target gene regulated by the p53 family and mediates the cell survival function of the basal physiologically relevant level of p53. We found that knockdown of BMP7 significantly attenuates the proliferation of p53-deficient breast cancer cells compared with the ones with wild-type p53. In
addition, we found that inhibitor of differentiation or DNA binding (Id)-2, a transcription factor required for cell survival, is regulated by the BMP7 and p53 pathways. Interestingly, whereas a functional BMP7 or p53 pathway is sufficient to maintain the basal level of Id2 expression, loss of both pathways abrogates Id2 expression. Furthermore, we showed that overexpression of Id2 can restore p53-deficient cells to survive in the absence of BMP7. As a result, we identified a previously unrecognized role of BMP7 in the maintenance of p53-deficient cell survival, at least in part, through Id2. We hypothesize that breast cancer patients with mutant p53 might benefit from targeted knockdown of BMP7 expression and/or targeted inhibition of the BMP7 pathway.

Materials and Methods

Cell culture. Breast cancer cell lines, MCF7 and MDA-MB-231, were cultured in DMEM (Invitrogen) supplemented with fetal bovine serum (Hyclone). MCF7 cells, which inducibly express p53, p53(R249S), p63, ΔNp63α, p63y, ΔNp63y, p73α, p73δ, or ΔNp73i, were cultured and induced as previously described (24–27). To generate inducible BMP7 knockdown cell lines with p53 or p21 stable knockdown, pcDNA3-Id2 was transfected into these cell lines as previously described (28). To generate inducible BMP7 knockdown cell lines with p3 or p21 stable knockdown, pbhve-H1-BMP7siRNA was cotransfected with pBabe-H1-p53siRNA or pBabe-H1-p21siRNA into these cell lines, in which a tetracycline repressor is expressed by pcDNA6. The siRNA expression vectors are described below. The resulting BMP7 and p53 or p21 dual-knockdown cell lines were selected with puromycin. p53 or p21 knockdown was confirmed by Western blot analysis and BMP7 knockdown was confirmed by Northern blot and Western blot analyses. To generate cell lines stably overexpressing Id2 with p53 stable knockdown, pcDNA3-Id2 was transfected into MCF7(p53-KD)-BMP7-KD cell lines. The resulting Id2 overexpression cell lines were selected with G418. Id2 overexpression was confirmed by Western blot analysis.

Plasmids. To generate a construct that expresses BMP7 siRNA under the control of tetracycline, one pair of oligos was cloned into pBabe-H1 at HinIII and BglII sites and the resulting construct was designated as pBabe-H1-BMP7siRNA. pbhve-H1 is a PolIII promoter–driven plasmid with a tetracycline repressor (Promega). Briefly, 0.25 μg of a luciferase reporter, 0.25 μg of pcDNA3 or pcDNA3 that expresses a p53 family protein, and 5 μg of Renilla luciferase (Promega) were cotransfected into H1299 cells. The fold increase in relative luciferase activity is a product of the luciferase activity induced by a p53 family protein divided by that induced by an empty pcDNA3 vector.

Luciferase assay. The dual luciferase assay was done in triplicate according to the manufacturer's instructions (Promega). Briefly, 0.25 μg of a luciferase reporter, 0.25 μg of pcDNA3 or pcDNA3 that expresses a p53 family protein, and 5 μg of Renilla luciferase report (Promega) were cotransfected into H1299 cells. The fold increase in relative luciferase activity is a product of the luciferase activity induced by a p53 family protein divided by that induced by an empty pcDNA3 vector.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation was done as previously described (15, 31). Briefly, MCF7 cells, which were untreated (−) or treated (+) with doxorubicin (0.25 μg/mL). To amplify the potential p53-RE from nt +2,839 to +3,031 in intron 1 of the BMP7 gene, PCR was done with the forward primer 5′-CCCCATGGCCGGAGACAGAAATGTTGG-3′ and the reverse primer 5′-AAGTGGAGAGGCGCTTCCTAGG-3′. To amplify the p53-RE from nt −2,312 to −2,131 in the p21 promoter, PCR was done with the forward primer 5′-CACATAGTCAGGCGCTTAC-3′ and the reverse primer 5′-TTCAGCTGAGAAGTGTCT-3′.

Colonization formation assay. MCF7 cells (400 well per well) and MDA-MB-231 cells (1,000 per well) were cultured in a six-well plate were cultured in the absence or presence of tetracycline (1.0 μg/mL) for 72 h, then untreated or treated with doxorubicin (0.025 μg/mL) for 4 h, followed by repetitive washes with DMEM to remove doxorubicin. The cells were maintained in fresh medium for the next 15 to 17 days, and then fixed with methanol/glacial acetic acid (7:1) and stained with 0.1% crystal violet.

Growth curve assay. MCF7 cells (4,000 well per well) and MDA-MB-231 cells (6,000 well per well) were cultured in six-well plates as described above. Cell culture medium was changed on day 3 and the number of cells was counted over a 7-day period.

Western blot analysis. Antibodies against p33 (FL-393), p21 (C-19), cyclin D1 (D6C-6), proliferating cell nuclear antigen (PCNA: PC10), N-Myc (C-20), Id1 (C-20), and Id2 (C-20) were purchased from Santa Cruz Biotechnology. Antibodies against BMP7 and p-SMA1/5/8 were purchased from Abcam and Cell Signaling Technology, respectively. Antibodies against hemagglutinin and Myc epitopes and actin were previously described (35).

Results

BMP7 is a novel target gene of the p53 family. To identify novel target genes regulated by the p53 family, the gene expression patterns were examined in MCF7 cells, which were untreated or induced to express p63y by a microarray study with the Affymetrix U133 plus GeneChip. We found that BMP7 was highly induced by p63y in MCF7 cells. To confirm this, Northern and Western blot analyses were done and BMP7 mRNA and protein were shown to
be induced by ΔNp63α, p63γ, and ΔNp63γ, but little, if any, by p63α (Fig. 1A, top, BMP7 mRNA; bottom, BMP7 protein). We also found that BMP7 was highly induced by p53, p73α, and ΔNp73α, but little, if any, by mutant p53(R249S) and p73α (Fig. 1B and C). The levels of p21 and GAPDH mRNA were examined as a loading control (Fig. 1A–C, bottom). The levels of actin protein were examined as a loading control (Fig. 1A–C, bottom). These results indicate that BMP7 is a common target gene of the p53 family.

DNA damage stabilizes and activates p53, leading to induction of p53 target genes. We reasoned that BMP7 as a p53 target gene is likely to be induced by DNA damage in cells that carry an endogenous wild-type p53 gene. To this end, the following two breast cancer cell lines were used: MCF7, in which endogenous p53 is wild-type, and MDA-MB-231, in which endogenous p53 is mutant. We found that BMP7 mRNA and protein were up-regulated in MCF7 cells treated with DNA-damaging agents, doxorubicin and etoposide, and nutlin-3, an inhibitor of murine double minute 2 (Fig. 1D, top, BMP7 mRNA; bottom, BMP7 protein). As a positive control, the expression of p21 mRNA was measured and found to be induced as expected (Fig. 1D, top). However, little, if any, up-regulation of BMP7 protein was observed in MDA-MB-231 cells treated with doxorubicin and etoposide, but not nutlin-3 (Fig. 1D, bottom), suggesting that p21 can be induced by DNA damage in a p53-independent manner.

The p53 family proteins regulate expression of their target genes through binding to specific DNA sequences in the promoter or intron. If BMP7 is transcriptionally regulated by the p53 family, one or more p53-REs should exist in the BMP7 gene. To test this, we analyzed the genomic locus encoding the BMP7 gene. As expected, we found one potential p53-RE located at nt +2,852 to +2,871 in intron 1 with the sequence GGACAAGCtGGAaCTTGTtT. On alignment with the consensus p53-RE [RRRC(A/T)(A/T)GYY; R represents purine and Y is pyrimidine], we found one mismatch in the first noncritical YYY stretch (Supplementary Fig. S1A, lowercase letter represents mismatch).

To determine whether the p53 family proteins transactivate BMP7 via the potential p53-RE, a 363-bp DNA fragment (nt +2,843–3,205) was cloned upstream of the minimal c-fos promoter in O-Fluc, a luciferase reporter (36). The resulting vector was designated as O-Fluc-BMP7 (Supplementary Fig. S1A). In addition, a mutant, in which four critical nucleotides in the potential p53-RE were altered, was also cloned into O-Fluc and designated as O-Fluc-BMP7-M (Supplementary Fig. S1A). The luciferase reporter under the control of the p21 promoter was used as a positive control (Supplementary Fig. S1B). Each of these luciferase reporters was cotransfected into H1299 cells with either a pcDNA3 control vector or a vector that expresses a p53 family protein. We found that the luciferase activity under the control of the potential p53-RE in the BMP7 gene was substantially increased by wild-type p53, p63γ, ΔNp63γ, p73α, p73β, and ΔNp73β, but little, if any, by mutant p53(R249S), ΔNp63α, and mutant ΔNp63γ(R419W) (Fig. 2A). In contrast, the luciferase activity for O-Fluc-BMP7-M was not significantly increased by various p53 family proteins (Fig. 2A). As a positive control, we showed that the luciferase reporter under the control of the p21 promoter was activated highly by p53, p63γ, and p73β; weakly by p63α, ΔNp63γ, and ΔNp73β; but not by p53(R249S), ΔNp63α, and mutant ΔNp63γ(R149W) (Fig. 2A). This suggests that the potential p53-RE in the BMP7 gene is a functional p53-RE.

Next, to determine whether the p53 family proteins bind to the p53-RE in the BMP7 gene in vivo, a chromatin immunoprecipitation assay was done with a pair of primers that amplify the region from nt +2,839 to +3,031 (Supplementary Fig. S1C). As a positive

Figure 1. Up-regulation of BMP7 by the p53 family. A to C, top. Northern blots were prepared with total RNAs isolated from MCF7 cells that were uninduced (−) or induced (+) to express p63α, ΔNp63α, p63γ, ΔNp63γ, p53, mutant p53(R249S), p73α, p73β, or ΔNp73β for 24 h. The blots were probed with cDNAs derived from the BMP7, p21, and GAPDH genes. The levels of p21 and GAPDH mRNA were measured as positive and loading controls, respectively. Bottom, Western blots were prepared with extracts from MCF7 cells treated as above, and then probed with antibodies against BMP7 and actin. D, Northern blots (top) and Western blots (bottom) were prepared with total RNAs and protein extracts from MCF7 and MDA-MB-231 cells that were untreated (−) or treated with 0.25 μg/mL of doxorubicin (DOX), 5 μM of nutlin-3, or 10 μM of etoposide (EPT) for 24 h. The blots were analyzed as in A.
control, the interaction of p53 with p53-RE1 in the p21 promoter was determined (Supplementary Fig. S1D). To analyze the binding of endogenous p53, MCF7 cells were untreated (−) or treated (+) with doxorubicin to activate p53, followed by cross-linking with formaldehyde and immunoprecipitation with anti-p53 or rabbit immunoglobulin G (IgG) as a negative control. We found that p53 bound to the p53-RE in the BMP7 gene as well as to the p53-RE1 in the p21 gene (Fig. 2B). To analyze the binding of exogenous p53, p63, or p73, MCF7 cells were uninduced (−) or induced (+) to express hemagglutinin-tagged p53, Myc-tagged p63γ/ΔNp63α, or hemagglutinin-tagged p73β/ΔNp73β, and then used for chromatin immunoprecipitation assay. The p63-DNA complexes were immunoprecipitated with anti-Myc antibody along with anti-Flag antibody as a control. The p53-DNA and p73-DNA complexes were immunoprecipitated with anti-hemagglutinin antibody along with anti-Flag antibody as a control. We found that exogenous p53, p63γ, p73β, and weakly to p63α and ΔNp63α, but not to mutant p53(R249S) and ΔNp63γ(R149W). The response of the p21 promoter to a p53 family protein was measured as a control. B to D, p53, p63γ, and p73β bind to the p53-RE in the BMP7 gene. p53-DNA complexes were captured with anti-p53 antibody, p63γ-DNA and p73β-DNA complexes were captured with anti-Myc and anti-hemagglutinin (HA) antibodies, respectively. Rabbit IgG and anti-Flag antibodies were used as a control. The binding of a p53 family protein to p53-RE1 in the p21 promoter was measured as a positive control.

Targeted disruption of BMP7 expression triggers proliferative defect in p53-deficient breast cancer cells. BMP7 is capable of stimulating and inhibiting cell proliferation, depending on the type and differentiation state of a cell (8, 9, 37). However, these studies were done in cells overexpressing BMP7 or treated with a high level of recombinant BMP7. To address the physiologic effect of BMP7 on cell proliferation, we generated MCF7 cell lines in which endogenous BMP7 can be inducibly knocked down by siRNA under the control of the tetracycline-regulated promoter. Two representative cell lines were shown in Fig. 3A. On induction of siRNA against BMP7, the levels of BMP7 transcript (Fig. 3A) and protein (Supplementary Fig. S2A) were markedly reduced. The levels of GAPDH transcript and actin protein were measured as loading controls. To examine whether BMP7 knockdown has an effect on MCF7 cell proliferation, MCF7-BMP7-KD-6 and MCF7-BMP7-KD-16 were chosen for colony formation assays in the absence or presence of treatment with doxorubicin for 4 h. We found that BMP7 knockdown alone had little effect on the colony-forming capability regardless of DNA damage (Fig. 3B, right). As a negative control, we found that tetracycline itself had no effect on MCF7 cell proliferation with or without doxorubicin treatment (Fig. 3B, left).

It was reported that in prostate and anaplastic thyroid carcinoma cells, the ability of BMP7 to regulate cell proliferation is mediated by p21 (8, 9). Thus, to determine whether p21 is involved, we generated MCF7 cell lines in which p21 was stably knocked down and BMP7 can be inducibly knocked down. Following treatment with doxorubicin, which activates p53 and subsequently induces p21, the levels of p21 in MCF7 cells were measured by Western blot analysis to examine the efficiency of p21 knockdown. As shown in Supplementary Fig. S2B (left), p21 was knocked down in clone nos. 33 and 41. Next, the efficiency of BMP7 inducible knockdown in the same MCF7 cell lines was measured by Northern blot and Western blot (Supplementary Fig. S2B, right). BMP7 mRNA and protein were efficiently knocked down in clone nos. 33 and 41 following treatment with tetracycline to induce siRNA against BMP7. Thus, p21 was stably knocked down and...
BMP7 can be inducibly knocked down in clone nos. 33 and 41, both of which were then used for colony formation assays. We found that on stable p21 knockdown, MCF7 cell proliferation was not affected by BMP7 knockdown regardless of DNA damage (Supplementary Fig. S2C).

Because BMP7 is a direct target of p53, we wanted to determine whether p53 plays a role in BMP7 function. Using a similar approach as described above, we generated multiple cell lines, including two cell lines (nos. 57 and 76) in which endogenous wild-type p53 is stably knocked down (Fig. 3C, left) and BMP7 (Fig. 3C, right; Supplementary Fig. S2D) can be inducibly knocked down by siRNA. Thus, MCF7(p53-KD)-BMP7-KD-57 and MCF7(p53-KD)-BMP7-KD-76 were chosen for colony formation assays. Surprisingly, we found that on stable knockdown of p53, cell proliferation was markedly inhibited by BMP7 knockdown regardless of treatment with doxorubicin (Fig. 3D). In addition, short-term growth curve assay showed that cell proliferation was inhibited by BMP7 knockdown when p53 was constitutively knocked down (Supplementary Fig. S2E). Collectively, these data indicate that BMP7 is required for MCF7 cell survival, which can be compensated by endogenous wild-type p53 but not by p21.

Given that overexpression of BMP7 and mutation of p53 occur frequently in primary breast tumors and cancer cell lines (11, 38), we examined whether BMP7 is required for cell proliferation in a breast cancer cell line in which endogenous p53 is mutant. For this purpose, MDA-MB-231 cell line was chosen because it carries an endogenous mutant p53(R280K) along with a high level of BMP7 (39). Multiple cell lines in which BMP7 can be inducibly knocked down were generated and three representatives were shown in Fig. 4A. On induction of siRNA against BMP7, the levels of BMP7 transcript (Fig. 4A) and protein (Supplementary Fig. S3) were markedly reduced. The levels of GAPDH transcript and actin protein were measured as loading controls. Clone nos. 19 and 36 were chosen for colony formation assays and clone no. 19 was also chosen for growth curve assay. We found that in the absence of functional p53, BMP7 was required for cell survival in MDA-MB-231 cells [Fig. 4B (right) and C]. As a control, tetracycline alone had no effect on cell proliferation for parental MDA-MB-231 cells (Fig. 4B, left). Thus, unlike wild-type p53 in MCF7 cells, mutant p53 in MDA-MB-231 cells is unable to compensate for the prosurvival function of BMP7.

Loss of Id2 expression is likely responsible for impaired survival of breast cancer cells with dysfunctional BMP7 and p53 pathways. To investigate the molecular mechanism by which BMP7 and wild-type p53 are involved in the cell survival process, we measured the expression of a group of genes that are required for cell proliferation and controlled by BMP7 and/or p53 signalings, including p21, cyclin D1, PCNA, phosphorylated Smad1/Smad5/Smad8 (p-Smad1/Smad5/Smad8), N-Myc, and Id1 and Id2. We found that on knockdown of BMP7 in MCF7 cells with wild-type p53, p21 expression was slightly increased whereas p53 expression was not affected (Fig. 5A, p53 and p21). In MCF7 cells with stable p53 knockdown, p53 was undetectable and, consequently, p21 was also not expressed regardless of BMP7 knockdown (Fig. 5A, p53

![Figure 3. Effect of BMP7 knockdown on cell proliferation in MCF7 cells. A, generation of MCF7 cell lines in which BMP7 can be inducibly knocked down. Northern blots were prepared with total RNAs isolated from MCF7 cells induced to express siRNA against BMP7 for 0 to 4 d and probed with cDNAs derived from the BMP7 and GAPDH genes. B, BMP7 knockdown alone had little effect on MCF7 cell proliferation regardless of doxorubicin treatment. Parental MCF7 and MCF7-BMP7-KD cells were uninduced or induced with tetracycline along with or without doxorubicin treatment (0.025 μg/mL), cultured for ~3 wks, and then fixed and stained to measure the number and size of colonies formed. C, left, identification of p53 knockdown cell lines. Cells were treated with doxorubicin (0.25 μg/mL) for 24 h, and the levels of p53 and actin proteins were detected by Western blot analysis. Right, identification of inducible BMP7 knockdown cell lines. Positive p53 knockdown clones were uninduced (−) and induced (+) with tetracycline to knock down BMP7 for 3 d, and then the levels of BMP7 transcript were measured by Northern blot analysis. D, cell proliferation was markedly inhibited by knockdown of BMP7 in p53-knockdown MCF7 cells regardless of doxorubicin treatment. The experiment was done as in B.](www.aacrjournals.org)
However, knockdown of BMP7 and/or p53 had little, if any, effect on the levels of cyclin D1 and PCNA expression (Fig. 5A, cyclin D1 and PCNA). Next, we examined the phosphorylation status of Smad1/Smad5/Smad8, which correlates with the status of BMP7 signaling. As expected, knockdown of BMP7 markedly inhibited Smad1/Smad5/Smad8 phosphorylation regardless of p53 status (Fig. 5A, p-Smad1/Smad5/Smad8). Similarly, expression of N-Myc, an oncoprotein (40) and a potential target of BMPs, was also inhibited by BMP7 knockdown (Fig. 5A, N-Myc). However, expression of Id1, a putative target of BMP7 (41, 42), was not significantly affected by knockdown of BMP7 and/or p53 (Fig. 5A, Id1). Interestingly, whereas expression of Id2, a common target of BMP7 and p53 (41–44), was weakly inhibited by stable p53 knockdown (Fig. 5A, Id2, compare lanes 1 and 3), its expression was abrogated by lack of both BMP7 and p53 (Fig. 5A, Id2, compare lanes 3 and 4). To further confirm this, the levels of p-Smad1/Smad5/Smad8 and Id2 were examined in MDA-MB-231 cells. Consistent with the data above, on BMP7 knockdown, the level of p-Smad1/Smad5/Smad8 was reduced (Fig. 5B, p-Smad1/Smad5/Smad8). Most importantly, the levels of Id2 protein and transcript were markedly decreased (Fig. 5B and C, Id2). Together, our data indicate that a functional BMP7 or p53 pathway is sufficient to maintain the basal levels of Id2 expression, and loss of both pathways abrogates Id2 expression. Thus, loss of Id2 expression is likely responsible for impaired survival of breast cancer cells with dysfunctional BMP7 and p53 pathways.

Overexpression of Id2 rescues proliferative defect induced by BMP7 knockdown in p53-deficient breast cancer cells. To further confirm that down-regulation of Id2 induced by BMP7 knockdown in p53-deficient breast cancer cells directly contributes to the proliferation defect, we generated five MCF7 cell lines in which Id2 is stably overexpressed along with p53 stable knockdown.
and BMP7 inducible knockdown (Fig. 6). Two representative cell lines, MCF7(p53/BMP7-KD)-Id2-5 and MCF7(p53/BMP7-KD)-Id2-6, were chosen for colony formation assay and clone no. 6 was also used for growth curve assay. Interestingly, we found that in MCF7 cells with stable p53 knockdown, the effect of BMP7 knockdown on cell proliferation was nearly abolished by stable overexpression of Id2 [Fig. 6B (compare the top wells with the bottom wells) and C]. This finding suggests that Id2 is a critical mediator of BMP7 and physiologically relevant levels of p53 in the proliferation of breast cancer cells.

Discussion

Here, we showed that BMP7 is a novel target gene regulated by the p53 family and is necessary for cell survival in p53-deficient breast cancer cells. In addition, we found that Id2, a transcription factor implicated for cell survival, is regulated by the BMP7 and p53 pathways. Interestingly, whereas a functional BMP7 or p53 pathway is sufficient to maintain the basal levels of Id2 expression, loss of both pathways abrogates Id2 expression. Furthermore, we showed that overexpression of Id2 can restore p53-deficient cells to survive in the absence of BMP7. As a result, we identified a previously unrecognized role for BMP7 in the maintenance of cell survival for p53-deficient cells, at least in part, through Id2 (Fig. 6D). Therefore, breast cancer patients might benefit from targeted repression of BMP7 expression and/or targeted inhibition of the BMP7 pathway because mutation of p53 and overexpression of BMP7 occur frequently in breast tumors.

Unlike p53, the functional pathways regulated by p63 and p73 are still poorly understood. In this study, we found that BMP7 can be induced by p63 and p73, especially their ΔN variants. We also found that both p63 and p73 isoforms directly bind to the p53-RE located at nt +2,852 to +2,871 in intron 1 of the BMP7 gene. The transcriptional regulation of BMP7 by p63 is consistent with, and/or might explain, a recent observation that the level of BMP7 transcript correlates with that of ΔNp63α in ectoderm during mouse development (45). Thus, it is possible that loss of p63 leads to diminished BMP7 expression, resulting in developmental defects in the p63-null mouse. In addition, the up-regulation of BMP7 by the ΔN variants of p63 and p73, both of which are frequently overexpressed in many types of cancer, may play a role in the prosurvival function of ΔNp63α and ΔNp73α. Therefore, further studies are needed to determine whether there is a correlation between BMP7 and ΔNp63α/ΔNp73α in primary breast tumor tissues.

The Id family proteins have four members, Id1, Id2, Id3, and Id4, and act as dominant negative inhibitors of basic helix-loop-helix transcription factors such as MyoD and E1A (4). Id proteins are found to play a role in the control of the cell cycle and mediate BMPs to modulate cell proliferation and differentiation (46, 47). Specifically, Id2 is found to play a crucial role in mammary gland development (48). In addition, lack of Id2 leads to a proliferation defect in the early stage of pregnancy in mice (49) whereas overexpression of Id2 stimulates cell proliferation in breast cancer cells (50). Moreover, a recent report showed that Id2 expression is induced by DNA damage in a p53-dependent manner (44). Consistent with this, we found that Id2 expression was markedly attenuated by BMP7 knockdown in p53-deficient cells, but only slightly by BMP7 or p53 knockdown in p53-proficient cells. Thus, BMP7 or wild-type p53 is sufficient to maintain the basal levels of Id2 expression, but loss of both pathways abrogates Id2 expression. In contrast, we found that expression of Id1, another target of BMP7, is not affected by double knockdown of BMP7 and p53 in MCF7 cells. Likewise, other cellular targets of BMPs and p53, such as cyclin D1, PCNA, N-Myc, and p21, were not found to be affected by knockdown of BMP7 and/or p53. Together, these data indicate that the basal levels of Id2, which is controlled by p53 and BMP7, correlate with the ability of breast cancer cells to survive and, thus, may be explored for breast cancer treatment.
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