Bone Morphogenetic Protein-focused Strategies to Induce Cytotoxicity in Lung Cancer Cells

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Abstract. *Background: High bone morphogenetic protein (BMP)-2 expression in lung carcinoma correlates with poor patient prognosis. The present study explored strategies to repress BMP signaling. Materials and Methods: The cytotoxicity of BMP2-knockdown, dorsomorphin derivatives, and microRNAs was tested in transformed and non-transformed lung cells. Microarray analyses of 1,145 microRNAs in A549 lung adenocarcinoma cells and two other transformed lung cell types relative to BEAS-2B bronchial epithelial cells were performed. Results: Reduced BMP2 synthesis inhibited A549 cell growth. The dorsomorphin derivative LDN-193189, but not DMH1 or DMH4, was strongly cytotoxic towards A549 cells, but not towards BEAS-2B cells. Microarray analysis revealed that 106 miRNAs were down-regulated and 69 miRNAs were up-regulated in the three transformed lines. Three down-regulated miRNAs, hsa-mir-34b, hsa-mir-34c-3p, and hsa-miR-486-3p, repressed a BMP2 reporter gene and were cytotoxic in A549 cells, but not towards BEAS-2B cells. Conclusion: The observed cytotoxicity suggests that reducing BMP signaling is a useful line of attack for therapy of lung cancer.*

Lung cancer is the leading cause of cancer-related deaths in the United States and the 5-year survival rate is an abysmal 15% (1). The potent growth factor bone morphogenetic protein-2 (BMP2) is often dysregulated in non-small cell lung cancer (NSCLC). Better understanding of how such growth factors influence lung cancer is sorely needed to develop new therapies.

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Normal mature lung cells do not produce BMP2, but several studies indicate that BMP2 plays a significant role in lung cancer. *BMP2* RNA and protein levels are abnormally elevated in lung tumors (2-4) and in serum from patients with lung cancer (5). BMP2 activates pro-oncogenic pathways (*e.g.*, phosphoinositide-3 kinase (PI3K)/ mammalian target of rapamycin (mTOR); SMAD1, 5/ DNA-binding protein inhibitor (ID)-1) and promotes lung tumor growth in mice (6-8). The BMP2 antagonist, noggin, reduces mixed metastatic lung cancer lesions in bone (9) and inhibits the growth of transformed lung cells in monolayer and in soft agar (10). Finally, variations in BMP2 levels (11) and specific *BMP2* gene polymorphisms (12) are associated with differences in patient survival and response to treatment. Therefore, blocking the pro-oncogenic signaling of BMP2 is a reasonable therapeutic strategy.

Analogs of the small organic molecule dorsomorphin have been shown to inhibit the type-1 BMP receptors with different degrees of selectivity (13-15). The dorsomorphin-derived analogs DMH1 and LDN-193189 (arbitrary compound designations) target the BMP type-I receptors activin A receptor type II-like 1 (ACVRL1/ALK1; HGNC:175), activin A receptor, type I (ACVR1/ALK2; HGNC:171), bone morphogenetic protein receptor, type IA (BMPR1A/ALK3; HGNC:1076), and bone morphogenetic protein receptor, type IB (BMPR1B/ALK6; HGNC:1077). LDN-193189 also inhibits the type-I transforming growth factor, beta receptor 1, TGFBR1/ALK5; HGNC:11772), the type-2 transforming growth factor, beta receptor II (TGFBR2; HGNC:11773), and the vascular endothelial growth factor A receptor-2 (KDR/VEGFR2; HGNC:6307). DMH4 inhibits VEGFR2 (13). SB-431542 is a chemically distinct inhibitor of TGFBR1 (16).

We have previously shown that highly malignant A549 lung adenocarcinoma cells express high levels of BMP2 (10). In contrast, BMP2 is practically undetectable in immortalized but non-tumorigenic BEAS-2B bronchial epithelial cells (10). We postulated that inhibitors of BMP

signaling should differentially affect these two cell types based on their differential BMP levels. We tested this hypothesis by measuring the influence of DMH1 and LDN-193189 on cell growth and survival in culture. We also assessed the influence of microRNAs (miRNAs) that repress the expression of BMP2.

Materials and Methods

Materials. Dorsomorphin analogs DMH1, DMH4, LDN-193189, SB-14373 were provided by Charles Hong (Vanderbilt University, Nashville, TN, USA). All except SB-14373 were re-suspended in the vehicle dimethyl sulfoxide (DMSO). SB-14373 was re-suspended in phosphate buffered saline (PBS). Cell culture and molecular reagents were from Sigma–Aldrich (St. Louis, MO, USA) and Invitrogen (Carlsbad, CA, USA). Ambion Pre-miR miRNA Precursors for hsa-mir-34b (PM12727), hsa-mir-34c-3p (PM12342), and hsa-miR-486-3p (PM12986) and Negative Control #1 (AM17100) were from Applied Biosystems (Austin, TX, USA).

Cell culture. A549 and non-transformed BEAS-2B cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, D5796, Sigma–Aldrich) supplemented with 5% fetal bovine serum and 2 mM glutamine. Cells were grown in 7% CO_2 at 37˚C. For experiments to test drugs, cells were plated in 96-well plates at a density of 2.0×10^4 cells per well. Drugs were added the following day. Plates were incubated for three days before performing the assays described below.

RNAi-mediated BMP2 knockdown. BMP2 (sc-270025-V; pooled viral particles with three BMP2-specific constructs encoding shRNAs) and Control (sc-108080) shRNA lentiviral particles were transduced according to the manufacturer's instructions (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

RNA isolation. Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions.

Reverse transcription – polymerase chain reaction (RT-PCR). Total RNA (1 μg) was reverse-transcribed using 100 units of SuperScript™ III RT (Invitrogen) and oligonucleotide (oligo) dT or random primers according to the manufacturer's instructions and 10% of the resulting cDNA was used for PCR. For *BMP2* PCR, primers spanned the second intron (exon 2 forward primer 5'-GAGTTGAGGCTGCTCAGCATGTT-3', exon 3 reverse primer 5'-TGGCATGGTTAGTGGAGTTCAG-3'). The predicted size of the amplified cDNA fragment is 868 bp, whereas a product generated from genomic *BMP2* gene would be 8639 bp. PCR conditions were: 95˚C, 2 min; followed by 25 or 30 cycles of 94˚C, 1 min; 55˚C, 1 min; 72˚C, 1 min; ending with 72˚C, 7 min. For peptidylprolyl isomerase A (CYPA/cyclophilin A; HGNC:9253), the forward primer was 5'-GCCATGGTCAACCCCACCGTG-3' and the reverse primer was 5'-CCACTGAGCACTGGAGAGAAAGGATTTGG-3'. The predicted size of the amplified cDNA fragment is 143 bp. PCR conditions were: 97˚C, 5 min; followed by 29 cycles of 94˚C, 30 s; 57˚C, 45 s; 72˚C, 1 min; ending with 72˚C, 2 min.

Enzyme linked immunosorbent assay (ELISA) for BMP2. Conditioned medium (CM) was pre-cleared by centrifugation at 4000 × *g* for 2 min at 4˚C to remove dead cells. Two milliliters pre-cleared CM was concentrated to about 100 μl using a Centricon Centrifugal Filter Unit with Ultracel YM-10 membrane (Millipore, Bedford, MA, USA) following the manufacturer's instruction. The Quantikine BMP2 Immunoassay ELISA kit (R&D system, Minneapolis, MN, USA) was used to quantify the BMP2 protein level in 50 μl concentrated CM following the manufacturer's instruction.

Reporter gene constructs. RLuc-empty (pSGG_3UTR_empty) and RLuc-3'UTR (pSGG_3UTR_BMP2, S208437) were described elsewhere (17). The SMAD binding element reporter was provided by Dr. Steve Harris, University of Texas Health Science Center, San Antonio, TX, USA. (18).

Reporter gene assays. Cells were plated and transfected with plasmid using FuGene6 Transfection Reagent (Roche, Indianapolis, IN, USA). Cells were lysed with 1×Passive Lysis Buffer (Promega, Madison, WI, USA) and luciferase activities were measured using the Luciferase Assay System (Promega). The luciferase activity of a co-transfected, constitutively expressed *Renilla* luciferase reporter plasmid was used to control for transfection efficiency. Protein concentration was used to control for cell density.

Methylene blue staining. Cells were fixed with 100% methanol. Fixative was removed and cells were stained with 1% methylene blue in 0.01 M borate buffer, pH 8.5, for 30 min (19). The stain was thoroughly washed with 0.01 M borate buffer, pH 8.5, until all excess stain was removed. Cells were photographed on a Gene Genius (Syngene, Frederick, MD, USA).

WST-1 cell proliferation assay. The Cell Proliferation Reagent WST-1 (Roche, Mannheim, Germany) is a colorimetric assay to quantify proliferation and cell viability after exposure to cytotoxic compounds in 96-well plates. Absorbance was measured at 450 nm using a Synergy HT, (Biotek, Winooski, VT, USA). WST-1 Reagent was added to culture medium without cells to control for background absorbance.

Lactate dehydrogenase (LDH) cytotoxicity assay. The Cytotoxicity Detection kit (LDH) (Roche) quantifies cell lysis by measuring LDH released from damaged cells into the media. Absorbance was measured at 490 nm using Synergy HT, (Biotek). LDH Reagent was added to culture medium without cells to control for background absorbance.

Statistical analyses. A Student's *t*-test (two-sample assuming equal variances) was used to evaluate differences. A nominal *p*-value of 0.05 was considered significant.

Results

RNAi-mediated inhibition of BMP2 synthesis inhibits cell growth. BMP2, but not other BMPs, is induced in NSCLC cells and cultured transformed lung cells (2-4, 10). Previous studies used the BMP antagonist noggin to test the influence of reduced BMP2 activity on transformation, tumorigenesis, and metastasis (9, 10). Because noggin binds many members of the BMP family (20), we used RNAi-mediated inhibition of BMP2 synthesis to specifically test if reduced BMP2 levels affect the behavior of transformed lung cells. We

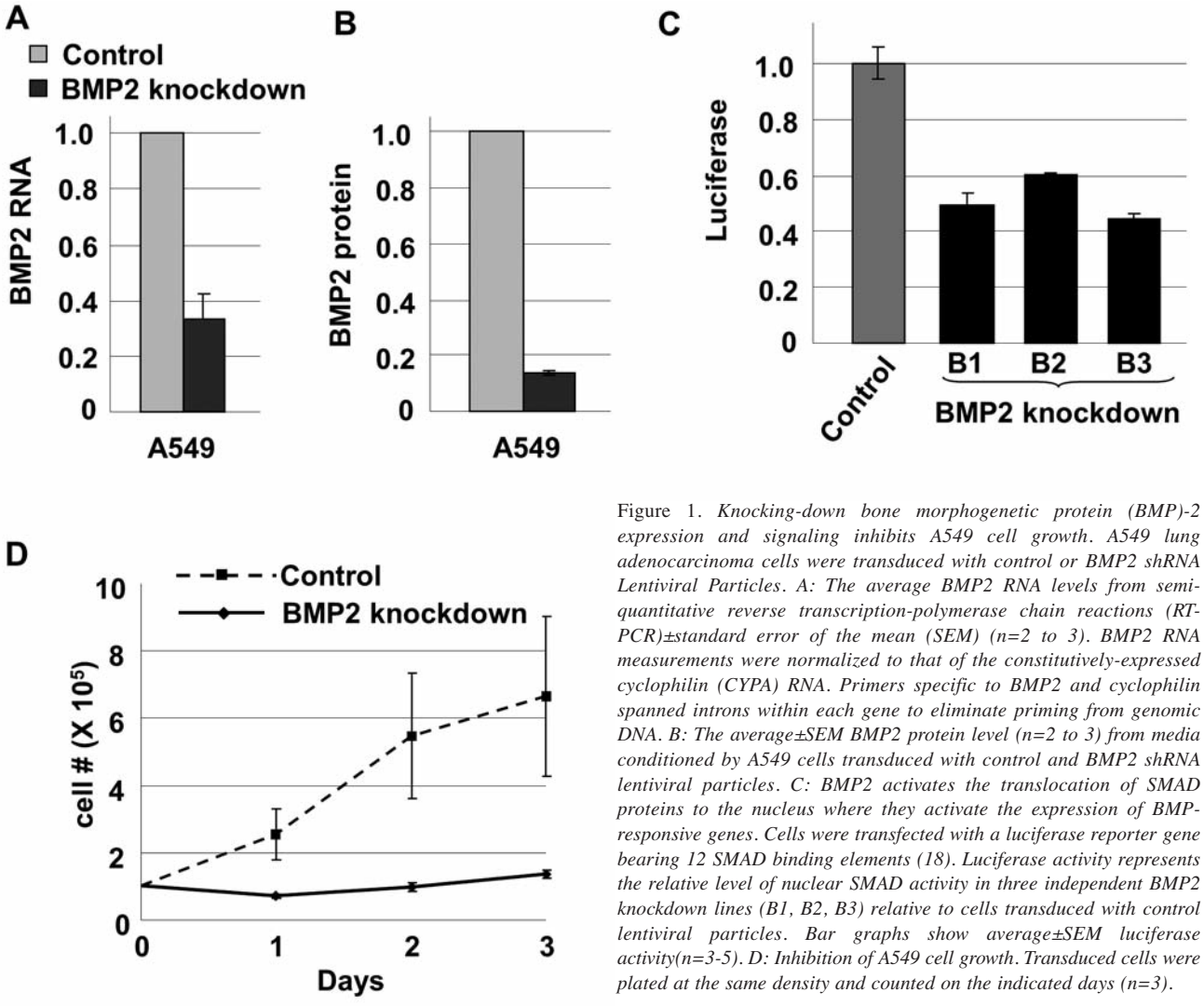


Figure 1. *Knocking-down bone morphogenetic protein (BMP)-2 expression and signaling inhibits A549 cell growth. A549 lung adenocarcinoma cells were transduced with control or BMP2 shRNA Lentiviral Particles. A: The average BMP2 RNA levels from semi-quantitative reverse transcription-polymerase chain reactions (RT-PCR)±standard error of the mean (SEM) (n=2 to 3). BMP2 RNA measurements were normalized to that of the constitutively-expressed cyclophilin (CYPA) RNA. Primers specific to BMP2 and cyclophilin spanned introns within each gene to eliminate priming from genomic DNA. B: The average±SEM BMP2 protein level (n=2 to 3) from media conditioned by A549 cells transduced with control and BMP2 shRNA lentiviral particles. C: BMP2 activates the translocation of SMAD proteins to the nucleus where they activate the expression of BMP-responsive genes. Cells were transfected with a luciferase reporter gene bearing 12 SMAD binding elements (18). Luciferase activity represents the relative level of nuclear SMAD activity in three independent BMP2 knockdown lines (B1, B2, B3) relative to cells transduced with control lentiviral particles. Bar graphs show average±SEM luciferase activity(n=3-5). D: Inhibition of A549 cell growth. Transduced cells were plated at the same density and counted on the indicated days (n=3).*

transduced A549 adenocarcinoma cells with *BMP2* and control shRNA lentiviral particles. The lentiviral particles effectively knocked-down *BMP2* mRNA levels (Figure 1A). BMP2 protein secreted into media conditioned by the A549 cells was reduced by 90% (Figure 1B).

BMP2 activates the translocation of SMAD proteins to the nucleus where they activate the expression of BMP-responsive genes. To test if BMP2 signaling was reduced, we transfected control and *BMP2* knock-down A549 cells with a luciferase reporter gene bearing 12 SMAD binding elements. Luciferase activity generated by this plasmid reports the relative level of nuclear SMAD activity (18). In three independent strains of BMP2 knock-down A549 cells, SMAD-dependent luciferase activity was about half that observed in control cells (Figure 1C). This confirms that reducing the synthesis of BMP2 reduces SMAD-dependent BMP2 signaling in these cells.

Finally, we compared the effect of *BMP2* knock-down on proliferation rates. The growth of A549 cells that were transduced with the *BMP2* shRNA lentiviral particles was reduced compared to control cells (Figure 1B). These results are consistent with BMP2 stimulating the rapid growth of transformed lung cells.

LDN-193189 reduced cell proliferation and is cytotoxic in A549 and BEAS-2B cells. LDN-193189 inhibits type I BMP receptors ALK1, -2, -3, and -6. LDN-193189 also inhibits the type-1 TGFβ receptor ALK5, and the type-2 TGFβ receptors, as well as VEGFR2. We hypothesized that LDN-193189 should reduce growth in A549 cells that express high levels of BMP2 but not in BEAS2B cells which do not express BMP2. After equal numbers of cells were plated in 96-well plates, we added increasing concentrations of drug dissolved in DMSO or an equal volume of DMSO as a vehicle control. To

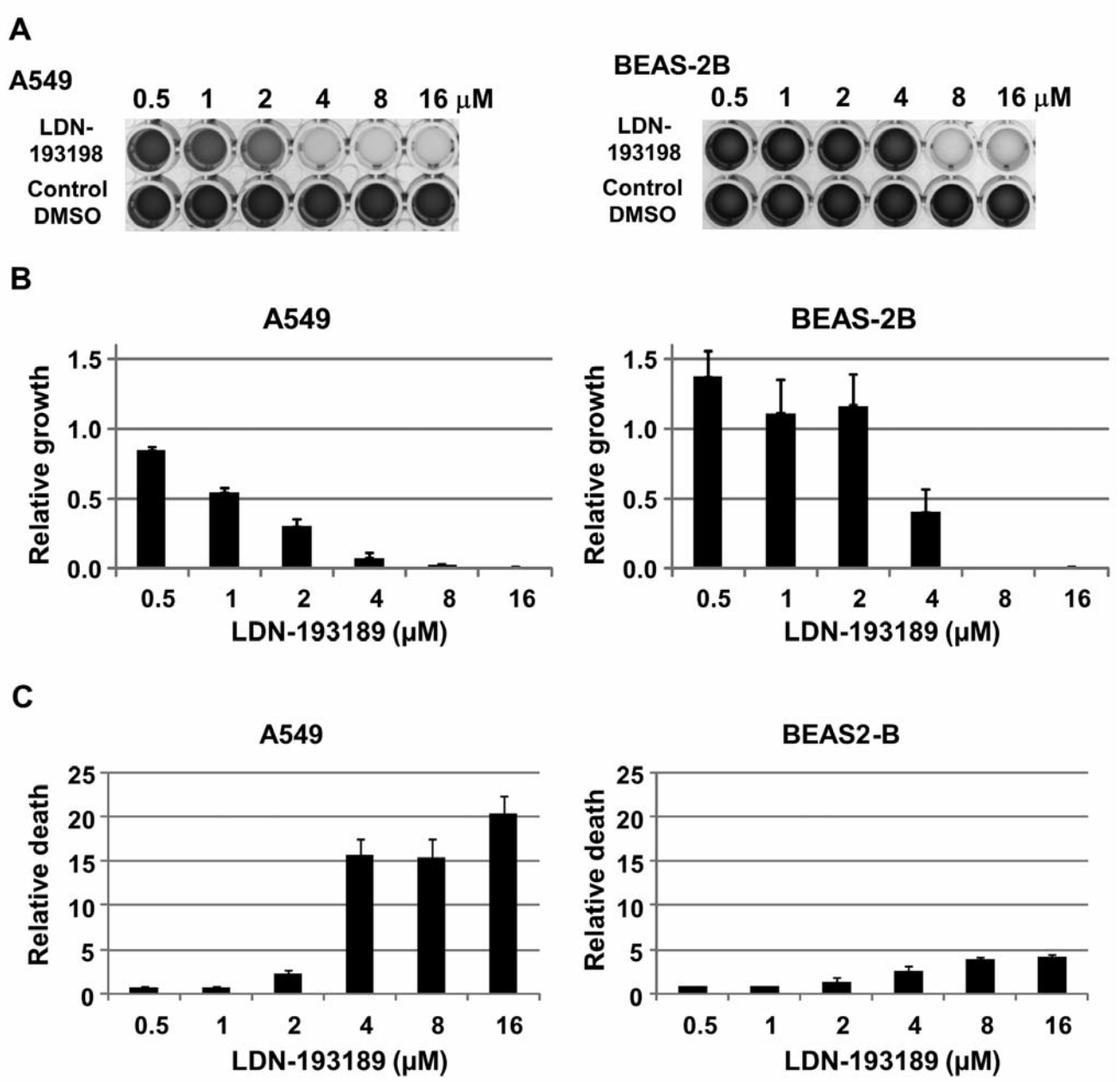


Figure 2. *The effect of compound LDN-193189 (inhibitor of ACVRL1, ACVR1, BMPR1A, BMPR1B, TGFBR1, TGFBR2, KDR receptors) on A549 and BEAS-2B cell growth and death. A: A total of 20,000 cells were plated, treated with drug dissolved in dimethyl sulfoxide (DMSO) at the indicated concentrations, and stained with methylene blue on the third day. Control cells were treated with equal volumes of DMSO. B: The relative number of metabolically-active cells in each well as indicated by the Roche WST-1 proliferation assay is shown as the mean±SEM. A549: n=5-14; BEAS2-B: n=5. C: The level of cytotoxicity as assessed by amount of lactate dehydrogenase (LDH) released into the three-day conditioned media relative to control (solvent) treated cells is shown as the mean±SEM. A549: n=6-15; BEAS-2B: n=6.*

avoid any influence of a drug on plating efficiency, cells were allowed to attach prior to drug addition. Cell density was assessed after three days by staining with methylene blue. The metabolic rate of the cells in each well was determined using the Roche WST-1 cell proliferation assay. Finally, to determine if any altered cell numbers were due to decreased cell proliferation or increased cell death, cytotoxicity was assessed using the Roche LDH Cytotoxicity Detection assay.

The number of cells was greatly reduced at the highest drug concentrations, as assessed by methylene blue staining (Figure

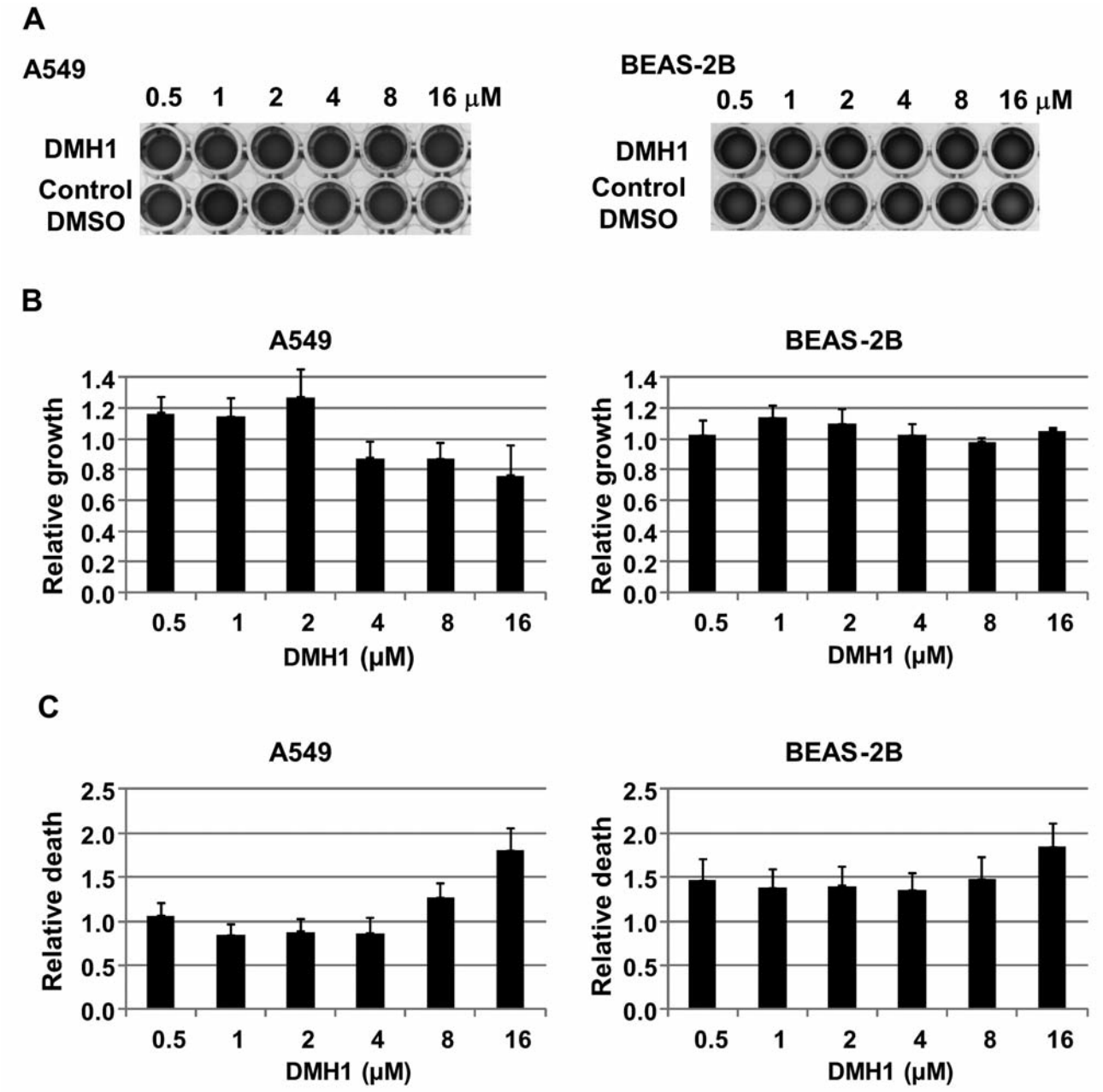
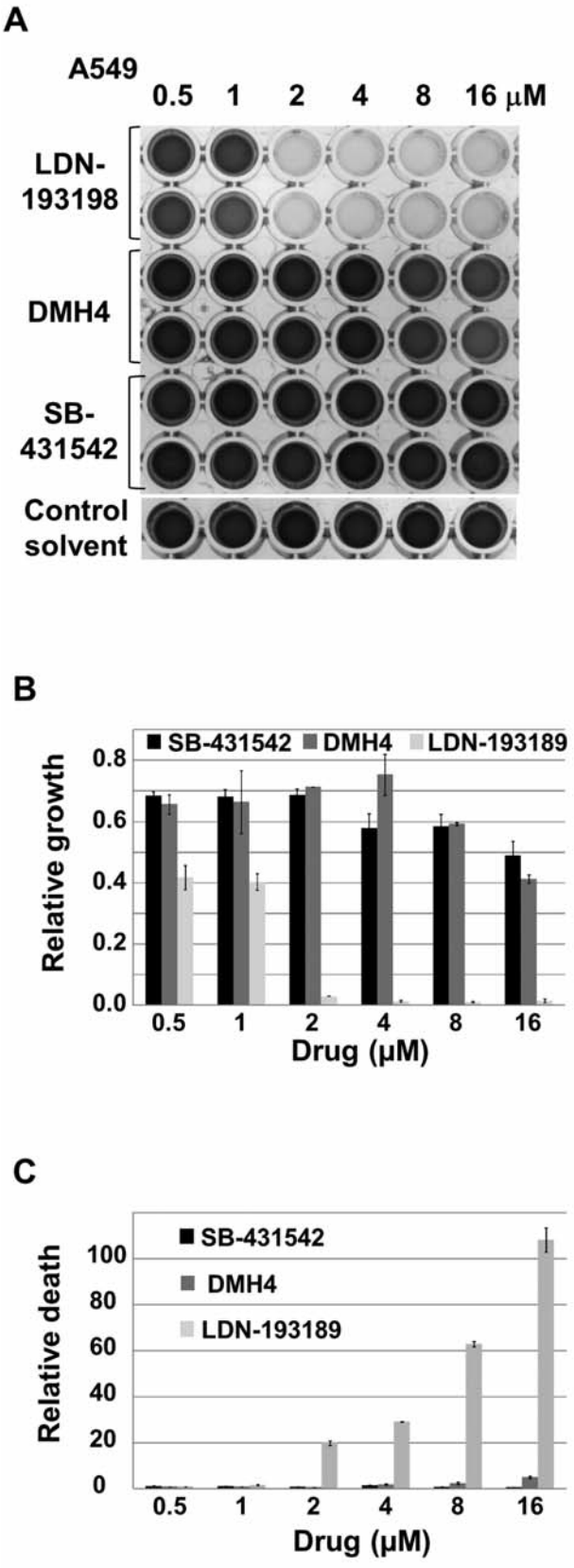


Figure 3. *The effect of compound DMH1 (inhibitor of ACVRL1, ACVR1, BMPR1A, BMPR1B receptors) on A549 and BEAS-2B cell growth and death. A. 20,000 cells were plated, treated with drug dissolved in dimethyl sulfoxide (DMSO) at the indicated concentrations, and stained with methylene blue on the third day. Control cells were treated with equal volumes of DMSO. B: The relative number of metabolically active cells in each well as indicated by the Roche WST-1 proliferation assay is shown as the mean±SEM. A549: n=8-11; BEAS2-B: n=5. C: The cytotoxicity as assessed by the amount of LDH released into the three-day conditioned media relative to control (solvent)-treated cells is shown as the mean±SEM. The only statistically significant difference from that of the control was observed with 16 μM DMH1 (A549: n=7-10, **p<0.004; BEAS-2B: n=6, *p<0.01).*

2A) and the WST-1 assay (Figure 2B). Both assays indicated that LDN-193189 reduced the growth of A549 cells at lower concentrations than in BEAS-2B cells. However, LDN-193189 was particularly cytotoxic against A549 cells, as indicated by a significant increase in LDH release (Figure 2C). At the highest concentration of drug (16 μM) there was 20-fold more LDH released than in the DMSO control. In contrast, only 4-fold more LDH was released from the BEAS-2B cells. Thus, in contrast to BEAS 2B cells that do not express BMP2, LDN-193189 was selectively cytotoxic to the BMP2-expressing A549 cells.



DMH1 had little effect on cell proliferation and cytotoxicity in A549 and BEAS2B cells. DMH1 targets the BMP type-I receptors ACVRL1, ACVR1, BMPR1A, and BMPR1B more selectively (14, 21, 22). Therefore, we tested the effect of DMH1 treatment on A549 and BEAS-2B cells. In contrast to LDN-193189, DMH1 failed to significantly inhibit the growth of either cell type, as assessed by both methylene blue staining and the WST1 assay (Figure 3A, B). In addition, DMH1 was not toxic to either cell type, except at its highest concentration (16 μM, Figure 3C).

TGFβ and VEGFR2 pathway inhibition do not account for the cytotoxic effects of LDN-193189. Because LDN-193189 also inhibits TGFBR1, TGFβR2, and VEGFR2, we tested selective inhibitors of these signaling paths. SB-431542 inhibits TGFβ signaling, *via* the activin A receptor, type IB (ACVR1B; HGNC:172), TGFBR1 and the activin A receptor, type IC, (ACVR1C; HGNC:18123) but not BMP signaling (16). DMH-4 is a selective inhibitor of VEGFR2. If the cytotoxic effects of LDN-193189 are due solely to inhibition of either pathway, then one or both of these drugs would produce similar antiproliferative and cytotoxic effects. We tested this hypothesis by performing a side-by-side drug assay by treating A549 cells with increasing concentrations of DMH4, SB-431542 and LDN-193189 (Figure 4). Methylene blue staining and the WST-1 and LDH release assays demonstrated that LDN-193189 had the strongest influence on A549 cells. In contrast, SB-431542 and DMH4 had little effect on A549 cells, except at the highest concentrations used. One remaining known difference between these drugs and LDN-193189 is that LDN-193189 inhibits TGFβR2. A549 cells do not express significant levels of TGFβ. Furthermore, because TGFβ blocks A549 cell growth (9, 23, 24), inhibiting TGFβ signaling would promote, not repress, A549 cell growth.

MicroRNAs that target BMP2 are cytotoxic. MiRNAs are small, ~21-22-nucleotide, single-stranded non-coding RNAs that commonly down-regulate protein synthesis by messenger RNA de-stabilization and translational repression

Figure 4. *The effect of compounds DMH4 (inhibitor of VEGFR2) and SB-431542 (inhibitor of TGFBR1) on A549 cell growth and death. A: A total of 20,000 cells were plated, treated with compound LDN-193189 or DMH4 dissolved in dimethyl sulfoxide (DMSO) or SB-431542 dissolved in phosphate buffered saline (PBS) at the indicated concentrations, and stained with methylene blue on the third day. Control cells were treated with equal volumes of DMSO or PBS. B: The relative number of metabolically-active cells in each well as indicated by the Roche WST-1 proliferation assay is shown as the mean±SEM, n=2. C: The level of cytotoxicity as assessed by the amount of LDH released into the three-day conditioned media relative to control (solvent) treated cells is shown as the mean±SEM, n=2.*

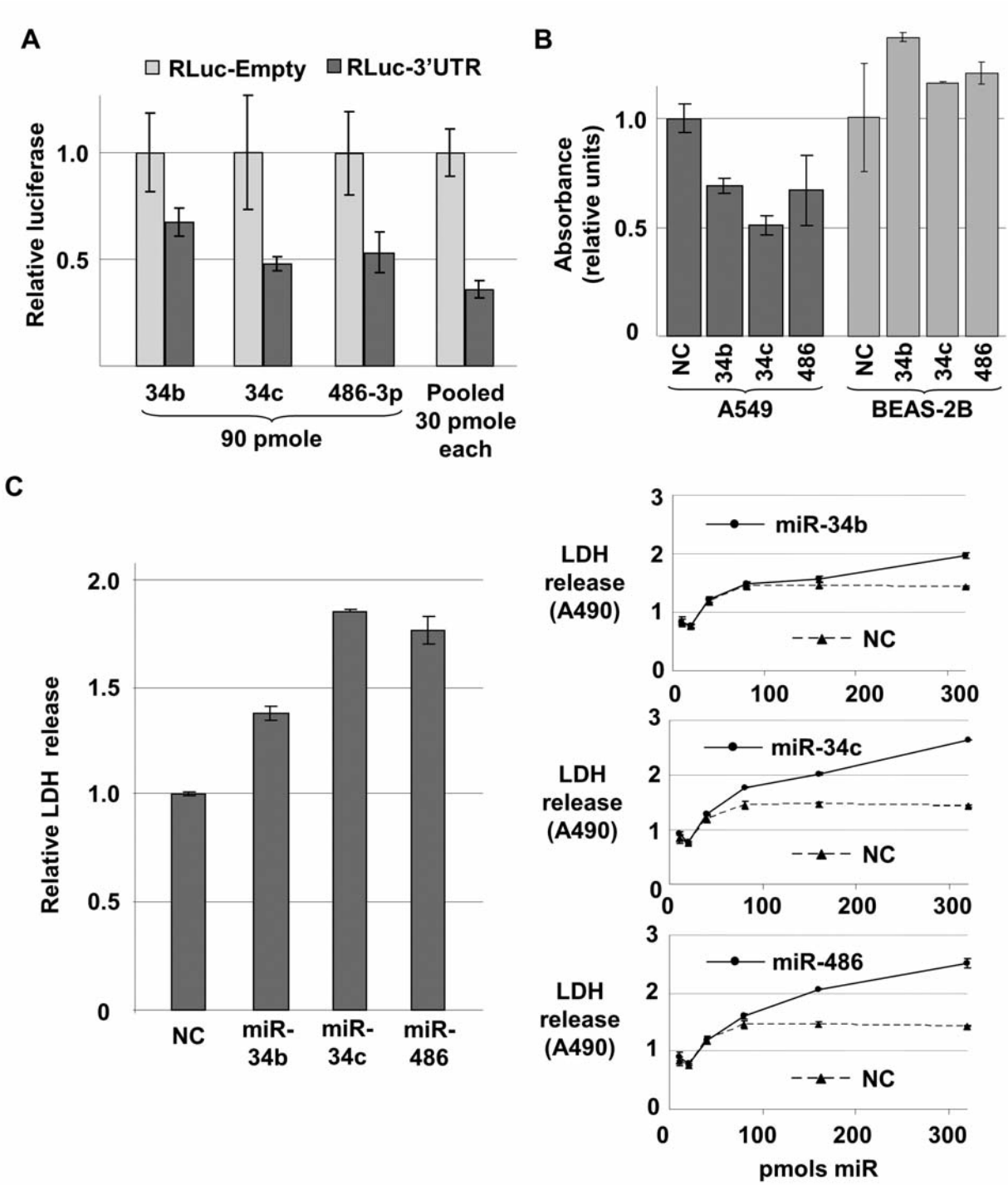


Figure 5. *MicroRNAs that inhibit bone morphogenetic protein (BMP)-2 expression are cytotoxic. A: A549 cells were co-transfected with a constitutive 60S ribosomal protein L10 (RPL-10) promoter-driven firefly luciferase reporter gene containing the human BMP2 3'UTR (RLuc-3'UTR) or without the 3'UTR (RLuc-Empty) and lacZ control. Ninety picomoles of each Ambion Pre-miR™ miRNA Precursors alone or a pool of 30 pmol each of miR-34b, -34c, and -486-3p or the Ambion Negative Control miRNA (NC) were co-transfected as indicated. All data were normalized to that of cells transfected with the Negative Control. Relative luciferase activity was measured at 24 h (n=4-9). B: A549 cell proliferation in the presence of 320 pmol miRNA precursors was measured four days after transfection using the Roche WST-1 cell proliferation. Similar studies in non-transformed BEAS-2B cells that express high levels of these microRNAs (Table I) failed to reveal any antiproliferative effect. C: The amount of lactate dehydrogenase (LDH) released by dying A549 cells into the medium was measured four days after transfection using the Roche LDH Cytotoxicity Detection assays. The bar graph shows the relative effect of each miRNA Precursor on LDH release at 320 pmoles. The line graphs show the average LDH released after transfection with increasing amounts of miRNA precursor (n=2).*

Table I. *Relative abundances of miR-34b, -34c, -486 in non-transformed BEAS-2B cells, in two mycoplasma-transformed BEAS-2B lines (BEAS^{tra1}, BEAS^{tra2}), and in A549 cells by Illumina v2 MiRNA Expression Profiling Assay (www.illumina.com/documents/products/workflows/workflow_microrna_assay). Bone Morphogenetic Protein (BMP) 2 is not detectable in non-transformed BEAS-2B cells, but is robustly expressed in transformed BEAS-2B and A549 cells (10, 28).*

	Cell line			
miRNA	BEAS-2B	BEAS^{tra1}	BEAS^{tra2}	A549
miR-34b	100	2	3	28
miR-34c-3p	100	3	3	31
miR-486-3p	100	25	57	12

(25-27). Using computational methods, we predicted that over 40 microRNAs may target 178 sites within the *BMP2* 3' untranslated region (UTR) (http://www.targetscan.org and http://pictar.bio.nyu.edu). To identify miRNAs that regulate BMP2 expression specifically in lung tumors, we used the Illumina v2 MiRNA Expression Profiling Assay (http://www.illumina.com/technology/miRNA_assay.ilmn; platform #GPL8179) to compare the abundance of miRNAs from BEAS-2B bronchial epithelial cells and three lines of neoplastic lung cells [A549, BEAS^{tra1}, and BEAS^{tra2} (10, 28)]. A549 cells were derived from a human alveolar cell carcinoma (29). In contrast, BEAS^{tra} cells were transformed by infecting the immortalized, but non-tumorigenic, BEAS-2B cell line with mycoplasma (10). Despite distinct origins, A549 and BEAS^{tra} cells are highly tumorigenic in immunocompromised mice. By examining the relative abundances of 1,145 miRNAs, we discovered that 106 miRNAs were down-regulated and 69 miRNAs were up-regulated in all three transformed lines (GEO accession # GSE557487). The abundance of several miRNAs predicted to interact with the BMP2 transcript inversely correlated with BMP2 secretion. That is, several miRNAs were expressed at high levels in normal BEAS-2B cells which do not synthesize any BMP2 but were significantly repressed in all three transformed lung cell lines which do synthesize BMP2 (10, 28). Such miRNAs may repress BMP2 synthesis in normal lung cells.

We analyzed three of the BMP2-targeting miRNAs repressed in malignant lung cells (Table I), previously shown to have interesting attributes relevant to lung cancer. For example, miR-34b and -34c are induced by p53 and are expressed at abnormally low levels in lung tumors (30-32). The level of miR-486 in sputum and serum of patients with lung adenocarcinoma differed from that of -healthy subjects and predicted patient survival (33, 34). We tested if these miRNAs repress BMP2 in A549 adenocarcinoma cells, by co-transfecting Ambion Pre-miR™ miRNA Precursor Molecules and a reporter gene driven by a constitutive ribosomal protein promoter and bearing the human *BMP2* 3'UTR (Figure 5A). These small, chemically-modified double-stranded RNA molecules mimic endogenous mature miRNAs. Mimics of miR-34b, -34c, and -486 activity significantly reduced the expression of the *BMP2* reporter gene bearing the *BMP2* 3'UTR (RLuc-3'UTR), but not the promoter-only construct (RLuc-empty). This demonstrates that the computer-predicted target sites in the *BMP2* 3'UTR are functional. Ninety picomoles of each miRNA alone or a pool consisting of 30 pmol of each miRNA inhibited reporter gene expression to comparable levels.

Because BMP2 promotes A549 cell growth and survival (Figure 1), (4, 6-8, 10), we then tested if miRNAs that target the *BMP2* mRNA alter A549 cell proliferation. A549 cells were transfected with Ambion mimics of miR-34b, -34c, and -486 or the Ambion Negative Control mimic. Four days after transfection, the WST-1 colorimetric assay and protein concentration were used to compare the number of proliferative cells in each situation. All three experimental miRNAs strongly reduced the number of A549 cells (Figure 5B). In contrast, growth of non-transformed BEAS-2B cells was not affected by these miRNAs (Figure 5B). Because the reduced number of cells may have resulted from decreased growth rate or increased cytotoxicity, we also assessed the amount of LDH released by dying cells into the media. The level of LDH inversely correlated with the decreased number of A549 cells following transfection with miR-34b, -34c, and -486 (compare Figure 4B and C).

Discussion

We demonstrated that an inhibitor of BMP signaling (LDN-193189) reduces the growth of A549 lung cancer cells, but not immortalized, non-transformed BEAS-2B cells. Thus, reducing BMP signaling is a valid therapeutic strategy for lung cancer. Secondly, we provide a global profile of miRNAs expressed in three transformed lung cancer lines relative to BEAS-2B cells. Finally, we demonstrate that restoring the level of three specific miRNAs whose expression is reduced in A549 cells (miR-34b, -34c, and -486) is cytotoxic. Understanding these inhibitory molecules may lead to strategies that pharmacologically re-awaken BMP2-repressive mechanisms in patients with lung cancer. Pursuing all therapeutic strategies is essential because existing lung cancer treatments are woefully inadequate.

An important aspect of drug development is the demonstration that normal cells are unaffected relative to tumor cells. We found that LDN-193189, an inhibitor of BMP signaling is more cytotoxic to A549 lung adenocarcinoma cells than to non-transformed BEAS-2B cells. While this work was in progress, LDN-193189 was reported to inhibit lung cancer cell growth and increase cell death, as assessed by different

methods (35). Our study complements the published study by testing cytotoxicity in non-transformed cells that do not express BMP2 (10). The induction of cell death, rather than inhibition of proliferation accounted for most of the cytotoxicity. In contrast to LDN-193189, more selective inhibitors of type I BMP, TGFβ, and VEGFRs exhibited little toxicity to either cell type. The limited cytotoxicity of DMH1, SB-431542, and DMH4 suggests that activating the receptors specific to these compounds is not sufficient to mediate the toxicity of LDN-193189. The ability of LDN-193189 to inhibit several pathways simultaneously may contribute to its effect on lung cancer cells. The precise mechanism by which LDN-193189 kills cells remains to be determined.

A sequence in the *BMP2* 3'UTR is 70% identical between mammals and fishes. We discovered that the function of this region in non-transformed lung cells differs from that in malignant cells (28). This sequence, which is targeted by the miRNAs tested here, is a potent repressor of gene expression in non-transformed BEAS-2B cells. In contrast, the sequence strongly stimulated BMP2 expression in A549 adenocarcinoma and mycoplasma-transformed BEAS-2B lung cells (28). Thus the ultra-conserved sequence functions as a post-transcriptional cis-regulatory switch. Three BMP2-targeting miRNAs are less abundant in strains of lung cells transformed by highly distinct means (Table I). The transformation-associated reduction in the abundance of miR-34b, -34c, and -486 miRNAs in transformed lung cells would impair the repression mediated by this switch and contribute to the elevated BMP2 levels observed in lung tumors (2-4, 10, 28).

The transcription of the bicistronic primary transcript bearing miR-34b and -34c is directly induced by the p53 tumor suppressor, and both miRNAs also inhibit proliferation in several cancer cell types (30-32). The list of experimentally-verified miR-34b and -34c targets is heavily weighted to genes that regulate cell behavior (36). We now demonstrate that BMP2, a growth factor that strongly influences proliferation, apoptosis, and differentiation, is also a target of miR-34b and -34c. MicroRNA-34b and -34c are most highly expressed in lung tissues (30). Inactivation of the *MIR-34b/c* gene and the subsequently dysregulated expression of *BMP2* and other influential genes in lung cells would profoundly influence the onset and progression of lung tumors.

Down-regulation of miR-486 also is associated with many types of human cancer, including human lung adenocarcinomas (37). A reduced level of miR-486 in sputum and serum is associated with lung cancer in diagnosis (33) and prognosis (34). Furthermore, the *MIR-486* gene resides within a region frequently deleted in cancer tissue (Chr 8p11) (38). We have now demonstrated that miR-486 has a repressive interaction with the *BMP2* 3'UTR and is cytotoxic to lung adenocarcinoma cells (Figure 5). The abnormally low abundance of tumor suppressive miRNAs such as miR-34b, -34c, and -486 may contribute to neoplastic cell behavior. In miRNA replacement therapy, miRNA mimics are used to restore these miRNAs, and most importantly their tumor-suppressor function, to normal levels. Restoring the natural level of these miRNAs may repress BMP2 synthesis and ameliorate the neoplastic behavior of lung cancer cells.

Acknowledgements

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References

1 AmericanCancerSociety. Cancer Facts and Figures 2013. Available from: http://www.cancer.org/acs/groups/content/@epidemiologysurveilance/documents/document/acspc-036845.pdf2013 [last accessed November 14].

2 Bieniasz M, Oszajca K, Eusebio M, Kordiak J, Bartkowiak J and Szemraj J: The positive correlation between gene expression of the two angiogenic factors: *VEGF* and *BMP-2* in lung cancer patients. Lung Cancer *66(3)*: 319-326, 2009.

3 Langenfeld EM, Bojnowski J, Perone J and Langenfeld J: Expression of bone morphogenetic proteins in human lung carcinomas. Ann Thorac Surg *80(3)*: 1028-1032, 2005.

4 Langenfeld EM, Calvano SE, Abou-Nukta F, Lowry SF, Amenta P and Langenfeld J: The mature bone morphogenetic protein-2 is aberrantly expressed in non-small cell lung carcinomas and stimulates tumor growth of A549 cells. Carcinogenesis *24(9)*: 1445-1454, 2003.

5 Hsu YL, Huang MS, Yang CJ, Hung, JY, Wu LY and Kuo PL: Lung tumor-associated osteoblast-derived bone morphogenetic protein-2 increased epithelial-to-mesenchymal transition of cancer by Runx2/Snail signaling pathway. J Biol Chem *286(43)*: 37335-37346, 2011.

6 Langenfeld EM, Kong, Y and Langenfeld J: Bone morphogenetic protein-2-induced transformation involves the activation of mammalian target of rapamycin. Mol Cancer Res *3(12)*: 679-684, 2005.

7 Langenfeld EM, Kong Y and Langenfeld J: Bone morphogenetic protein 2 stimulation of tumor growth involves the activation of Smad-1/5. Oncogene *25(5)*: 685-692, 2006.

8 Langenfeld EM and Langenfeld J: Bone morphogenetic protein-2 stimulates angiogenesis in developing tumors. Mol Cancer Res *2(3)*: 141-149, 2004.

9 Feeley BT, Liu NQ, Conduah AH, Krenek L, Roth K, Dougall WC, Huard J, Dubinett S and Lieberman JR: Mixed metastatic lung cancer lesions in bone are inhibited by noggin overexpression and rank:Fc administration. J Bone Miner Res *21(10)*: 1571-1580, 2006.

10 Jiang S, Zhang S, Langenfeld J, Lo SC and Rogers MB: Mycoplasma infection transforms normal lung cells and induces bone morphogenetic protein 2 expression by post-transcriptional mechanisms. J Cell Biochem *104(2)*: 580-594, 2007.

11 Beer DG, Kardia SL, Huang CC, Giordano TJ, Levin AM, Misek DE, Lin L, Chen G, Gharib TG, Thomas DG, Lizyness ML, Kuick R, Hayasaka S, Taylor JM, Iannettoni MD, Orringer MB and Hanash S: Gene-expression profiles predict survival of patients with lung adenocarcinoma. Nat Med *8(8)*: 816-824, 2002.

12 Lin M, Stewart DJ, Spitz MR, Hildebrandt MA, Lu C, Lin J, Gu J, Huang M, Lippman SM and Wu X: Genetic variations in the transforming growth factor-beta pathway as predictors of survival in advanced non-small cell lung cancer. Carcinogenesis *32(7)*: 1050-1056, 2011.

13 Hao J, Ho JN, Lewis JA, Karim KA, Daniels RN, Gentry PR, Hopkins CR, Lindsley CW and Hong CC: *In vivo* structure-activity relationship study of dorsomorphin analogues identifies selective VEGF and BMP inhibitors. ACS Chem Biol *5(2)*: 245-253, 2010.

14 Cuny GD, Yu PB, Laha JK, Xing X, Liu JF, Lai CS, Deng DY, Sachidanandan C, Bloch KD and Peterson RT: Structure-activity relationship study of bone morphogenetic protein (BMP) signaling inhibitors. Bioorg Med Chem Lett *18(15)*: 4388-4392, 2008.

15 Yu PB, Deng DY, Lai CS, Hong CC, Cuny GD, Bouxsein ML, Hong DW, McManus PM, Katagiri T, Sachidanandan C, Kamiya N, Fukuda T, Mishina Y, Peterson RT and Bloch KD: BMP type I receptor inhibition reduces heterotopic [corrected] ossification. Nat Med *14(12)*: 1363-1369, 2008.

16 Inman GJ, Nicolas FJ, Callahan JF, Harling JD, Gaster LM, Reith AD, Laping NJ and Hill CS: SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol Pharmacol *62(1)*: 65-74, 2002.

17 Kruithof BP, Fritz DT, Liu Y, Garsetti DE, Frank DB, Pregizer SK, Gaussin V, Mortlock DP and Rogers MB: An autonomous BMP2 regulatory element in mesenchymal cells. J Cell Biochem *112*: 666-674, 2010.

18 Zhao M, Harris SE, Horn D, Geng Z, Nishimura R, Mundy GR and Chen D: Bone morphogenetic protein receptor signaling is necessary for normal murine postnatal bone formation. J Cell Biol *157(6)*: 1049-1060, 2002.

19 Oliver HO, HN, Bishop JE, Cole PJ and Laurent GJ: A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. Cell Science *92*: 513-518, 1989.

20 Gazzerro E and Minetti C: Potential drug targets within bone morphogenetic protein signaling pathways. Curr Opin Pharmacol *7(3)*: 325-333, 2007.

21 Hao J, Daleo MA, Murphy CK, Yu PB, Ho JN, Hu J, Peterson RT, Hatzopoulos AK and Hong CC: Dorsomorphin, a selective small molecule inhibitor of BMP signaling, promotes cardiomyogenesis in embryonic stem cells. PLoS One *3(8)*: e2904, 2008.

22 Yu PB, Hong CC, Sachidanandan C, Babitt JL, Deng DY, Hoyng SA, Lin HY, Bloch KD and Peterson RT: Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol *4(1)*: 33-41, 2008.

23 Roberts AB, Anzano MA, Wakefield LM, Roche NS, Stern DF and Sporn MB: Type beta transforming growth factor: a bifunctional regulator of cellular growth. Proc Natl Acad Sci USA *82(1)*: 119-123, 1985.

24 Robinson, SP and Rose, WC: Transforming growth factor beta 1: lack of *in vivo* antitumor activity on A549 and Wehi 3BD+ tumors. Anticancer Res *12(1)*: 73-79, 1992.

25 Jing Q, Huang S, Guth S, Zarubin T, Motoyama A, Chen J, Di Padova F, Lin SC, Gram H and Han J: Involvement of microRNA in AU-rich element-mediated mRNA instability. Cell *120(5)*: 623-634, 2005.

26 Wu L, Fan J and Belasco JG: MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci USA *103(11)*: 4034-4039, 2006.

27 Bartel DP: MicroRNAs: target recognition and regulatory functions. Cell *136(2)*: 215-233, 2009.

28 Jiang S, Fritz DT and Rogers MB: A conserved post-transcriptional BMP2 switch in lung cells. J Cell Biochem *110(2)*: 509-521, 2010.

29 Lieber M, Smith B, Szakal A, Nelson-Rees W and Todaro G: A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int J Cancer *17(1)*: 62-70, 1976.

30 Bommer GT, Gerin I, Feng Y, Kaczorowski AJ, Kuick R, Love RE, Zhai Y, Giordano TJ, Qin ZS, Moore BB, MacDougald OA, Cho KR and Fearon ER: p53-mediated activation of miRNA34 candidate tumor-suppressor genes. Curr Biol *17(15)*: 1298-1307, 2007.

31 Corney DC, Flesken-Nikitin A, Godwin AK, Wang W and Nikitin AY: MicroRNA-34b and MicroRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. Cancer Res *67(18)*: 8433-8438, 2007.

32 He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, Xue W, Zender L, Magnus J, Ridzon D, Jackson AL, Linsley PS, Chen C, Lowe SW, Cleary MA and Hannon GJ: A microRNA component of the p53 tumour suppressor network. Nature *447(7148)*: 1130-1134, 2007.

33 Yu L, Todd NW, Xing L, Xie Y, Zhang H, Liu Z, Fang H, Zhang J, Katz RL and Jiang F: Early detection of lung adenocarcinoma in sputum by a panel of microRNA markers. Int J Cancer *127*: 2870-2878, 2010.

34 Hu Z, Chen X, Zhao Y, Tian T, Jin G, Shu Y, Chen Y, Xu L, Zen, K, Zhang C and Shen H: Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer. J Clin Oncol *28(10)*: 1721-1726, 2010.

35 Langenfeld E, Deen M, Zachariah E and Langenfeld J: Small molecule antagonist of the bone morphogenetic protein type I receptors suppresses growth and expression of Id1 and Id3 in lung cancer cells expressing Oct4 or nestin. Mol Cancer *12(1)*: 129, 2013.

36 Hermeking H: The miR-34 family in cancer and apoptosis. Cell Death Differ *17(2)*: 193-199, 2009.

37 Navon R, Wang H, Steinfeld I, Tsalenko A, Ben-Dor A and Yakhini Z: Novel rank-based statistical methods reveal microRNAs with differential expression in multiple cancer types. PLoS One *4(11)*: e8003, 2009.

38 Oh HK, Tan AL, Das K, Ooi CH, Deng NT, Tan IB, Beillard E, Lee J, Ramnarayanan K, Rha SY, Palanisamy N, Voorhoeve PM, and Tan P: Genomic Loss of miR-486 Regulates Tumor Progression and the OLFM4 Antiapoptotic Factor in Gastric Cancer. Clin Cancer Res *17(9)*: 2657-2667, 2011.

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