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A Conserved Post-Transcriptional BMP2 Switch in Lung Cells

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ABSTRACT

An ultra-conserved sequence in the bone morphogenetic protein 2 (BMP2) 3′ untranslated region (UTR) markedly represses *BMP2* expression in non-transformed lung cells. In contrast, the ultra-conserved sequence stimulates *BMP2* expression in transformed lung cells. The ultra-conserved sequence functions as a post-transcriptional *cis*-regulatory switch. A common single-nucleotide polymorphism (SNP, rs15705, +A1123C), which has been shown to influence human morphology, disrupts a conserved element within the ultra-conserved sequence and altered reporter gene activity in non-transformed lung cells. This polymorphism changed the affinity of the *BMP2* RNA for several proteins including nucleolin, which has an increased affinity for the C allele. Elevated BMP2 synthesis is associated with increased malignancy in mouse models of lung cancer and poor lung cancer patient prognosis. Understanding the *cis*- and *trans*-regulatory factors that control BMP2 synthesis is relevant to the initiation or progression of pathologies associated with abnormal BMP2 levels. J. Cell. Biochem. 110: 509–521, 2010.

KEY WORDS: CANCER; GENE REGULATION; GROWTH FACTOR; SNP, SINGLE-NUCLEOTIDE POLYMORPHISM; NUCLEOLIN; POST-TRANSCRIPTIONAL; LUNG

Lung cancer is the leading cause of cancer death in the United States. An estimated 16,390 people died from lung cancer in 2007 in the United States [American Cancer Society, 2007]. Although smoking accounts for most cases, the pathogenic mechanisms leading to lung cancer are largely unknown. Due to a lack of effective treatment, the 5-year survival rate is only 15% in lung cancer patients [American Cancer Society, 2007]. As in most cancers, many genes in essential developmental pathways are misregulated in lung cancer cells [Daniel et al., 2006]. These include a potent morphogen, bone morphogenetic protein 2 (BMP2) [Langenfeld et al., 2003, 2005a]. Understanding the genetic mechanisms that regulate key factors like BMP2 is relevant to patient prognosis and to developing new therapies for lung cancer.

BMP2 AND CANCER

BMP2 is a multi-functional cytokine that belongs to the transforming growth factor-β (TGF-β) superfamily [Chen et al., 2004]. BMP2 regulates many essential cellular processes in development including cell proliferation, apoptosis, differentiation, cell-fate determination, and morphogenesis. Aberrant BMP2 levels were reported in lung, breast, colon, prostate, and pancreatic cancers [Harris et al., 1994; Reinholz et al., 2002; Langenfeld et al., 2003, 2005a; Hardwick et al., 2004]. BMP2 plays different tumorigenic roles in tumors from different tissues [Kleeff et al., 1999; Ghosh-Choudhury et al., 2000a,b; Reinholz et al., 2002; Langenfeld et al., 2003, 2005a; Hardwick et al., 2004; Horvath et al., 2004; Raida et al., 2005]. *BMP2* is highly induced in all types of lung carcinomas as compared to normal tissues or benign lung tumors [Langenfeld et al., 2005a]. Elevated BMP2 levels in lung tumors appear to promote malignant progression by increasing cell proliferation and migration and by stimulating angiogenesis [Langenfeld et al., 2003, 2006; Langenfeld and Langenfeld, 2004]. Furthermore, the BMP antagonist, noggin, reduced metastasis to lungs and bones in two mouse models of lung cancer [Feeley et al., 2006; Langenfeld et al., 2006]. We previously reported that mycoplasma stimulates *BMP2* expression in human lung cells [Jiang et al., 2008]. Furthermore,

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chronic mycoplasma infection promotes the oncogenic transformation of immortalized bronchial epithelial cells (BEAS-2B) as determined by the ability to form colonies in soft agar and tumors in nude mice [Jiang et al., 2008]. Misregulation of *BMP2* expression could significantly influence the onset and progression of lung cancer and other human diseases. Indeed, a high level of *BMP2* mRNA in non-small cell lung carcinomas (NSCLC) was found to negatively predict survival [Beer et al., 2002]. Understanding the abnormal up-regulation of *BMP2* in lung cancer cells will increase our understanding of this devastating disease.

BMP2 GENE REGULATION

Similar to other potent cytokines, *BMP2* expression is controlled tightly by both positive and negative regulation at many levels [Helvering et al., 2000; Ghosh-Choudhury et al., 2001; Canalis et al., 2003; Abrams et al., 2004; Fritz et al., 2004, 2006; Xu and Rogers, 2007]. Accumulating evidence indicates that the concentration of potent growth factors like BMP2 is limited by modulating mRNA translatability and metabolism [Kuersten and Goodwin, 2003; Wilusz and Wilusz, 2004]. We discovered that the 3′ untranslated region (3′UTR) of the *BMP2* mRNA contains an ultra-conserved sequence that ranks among the most evolutionarily conserved sequences described [Abrams et al., 2004; Fritz et al., 2004]. The nearly 300 nt of the *BMP2* 3′UTR that is 73% conserved between mammals and fish (450 million years of separation) is unique to the *BMP2* mRNA [Fritz et al., 2004; Hu et al., 2006]. The ultra-conserved sequence contains several conserved AU-rich elements (AREs) which are well-studied sequence motifs that regulate mRNA stability and translation [Kuersten and Goodwin, 2003; Wilusz and Wilusz, 2004]. The presence of the conserved ARE motifs suggests that *BMP2* gene regulation involves post-transcriptional mechanisms. Indeed, we previously reported that the ultra-conserved sequence of the *BMP2* 3′UTR regulates *BMP2* mRNA half-life in vitro and up-regulates *BMP2* reporter genes in tissue culture [Fritz et al., 2004, 2006]. We also showed that a common single-nucleotide polymorphism (SNP) rs15705 that disrupts a conserved ARE altered the binding of proteins to the *BMP2* RNA and the rate of in vitro RNA decay [Fritz et al., 2006; Devaney et al., 2009]. More importantly, this SNP is associated with measurable differences in fat, muscle, and bone mass in humans [Devaney et al., 2009]. Because understanding the proteins that bind to the *BMP2* 3′UTR will provide insights into the regulatory mechanisms that control *BMP2* gene expression, we have begun to identify the proteins that interact with the polymorphic region.

BMP2 mRNA and protein are absent or barely detectable in non-transformed BEAS-2B (immortalized human bronchial epithelial) cells [Jiang et al., 2008]. In contrast, transformed BEAS-2B and A549 (human lung adenocarcinoma) cells express a high level of *BMP2* mRNA and protein [Jiang et al., 2008]. The differential levels of *BMP2* expression in these cell lines mirror *BMP2* levels in normal and malignant lung tissues [Langenfeld et al., 2005a]. We have used these cell culture models and transgenic mice to show, *for the first time*, that the ultra-conserved sequence is a post-transcriptional regulatory switch that mediates *BMP2* down-regulation in normal lung cells and up-regulation in transformed lung cells. We also demonstrate that the rs15705 allele alters the affinity of the BMP2 RNA for the endogenous nucleolin protein and reporter gene activity in human lung cells. Our data provide mechanistic evidence regarding how the rs15705 polymorphisms in this post-transcriptional regulatory element modulate BMP2 synthesis. Pharmacological agents that interact with the factors that bind the ultra-conserved sequence may influence the onset and course of BMP2-sensitive pathologies.

MATERIALS AND METHODS

CELL CULTURE

A549 and non-transformed BEAS-2B cells were obtained from Dr. John Langenfeld at Robert Wood Johnson Medical School (New Brunswick, NJ). BEAS-2B cells were infected and transformed with mycoplasma as described in Jiang et al. [2008]. All cells were cultured in Dulbecco's modified Eagles medium (D5796, Sigma–Aldrich, St. Louis, MO) with 5% fetal bovine serum and 2 mM glutamine in 5% CO_2 at 37°C.

RNA ISOLATION

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

REVERSE TRANSCRIPTION-PCR (RT-PCR)

One microgram of total RNA was reverse transcribed using 200 units of SuperScript™ III RT (Invitrogen) and oligonucleotide (oligo) dT or random primers according to the manufacturer's instructions. Ten percent 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 DNA would be 8,639 bp. For luciferase PCR, primers spanned the 67 bp SV40 intron in the pGL2 basic vector (primer A 5′-GATCCTCATAAAGGCCAAGAAG-3′, primer B 5′-CTTGTATAGCAGTGCAGC-3′). The predicted size of the amplified cDNA fragment is 533 bp, whereas a product generated from plasmid DNA would be 600 bp. Primer C (5′-TCTGACCATTATACTTCATGTG-3′) used in Figure 3 is in the ultra-conserved sequence. The condition for the *BMP2* and luciferase PCR reactions was: 95°C for 2 min; followed by 25 or 30 cycles of 94°C for 1 min; 55°C for 1 min; 72°C for 1 min; ending with 72°C for 7 min. For β-actin PCR, primers spanned the second intron (exon 2 primer 5′-CGTGGGGCGCCCCAGGCACCA-3′, exon 3 primer 5′-TTGGCCTTAGGGTTCAGGGGGG-3′). The predicted size of the amplified cDNA fragment is 242 bp, whereas a product generated from genomic DNA would be 376 bp. The conditions for the β-actin reactions were: 95°C for 2 min; followed by 30 cycles of 95°C for 1 min; 60°C for 1 min; 72°C for 1 min; ending with 72°C for 7 min. PCR reactions were analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

ELISA

Conditioned medium (CM) was pre-cleared by centrifugation at 4,000*g* for 2 min at 4°C to remove dead cells. Two milliliters of

pre-cleared CM was concentrated to about 100 μl using a Centricon Centrifugal Filter Unit with Ultracel YM-10 membrane (Millipore, Bedford, MA) following the manufacturer's instruction. The Quantikine BMP2 Immunoassay ELISA kit (R&D System, Minneapolis, MN) was used to quantify the BMP2 protein level in 50 μl of concentrated CM following the manufacturer's instruction.

TRANSGENIC MICE

All animals were handled in accordance with the Guidelines for Care and Use of Experimental Animals and approved by the New Jersey Medical School Institutional Animal Care and Use Committee (Protocol Nos. 04086 and 00100). The transgenic mouse strain bearing an intact *BMP2*-controlled *lacZ* expressing transgene was described in Fritz et al. [2006]. The β-gal expression pattern of the reporter gene in transgenic mice was detected by X-Gal staining [Agah et al., 1997]. The mice expressing Cre-recombinase driven by the CMV promoter [Schwenk et al., 1995] were kindly provided by Dr. Richard Eckner at UMDNJ, New Jersey Medical School.

MOUSE BMP2 REPORTER GENE CONSTRUCTS

Luciferase reporter constructs (Fig. 1, constructs A, B, C, D) containing the mouse *BMP2* sequences were generated as described in Fritz et al. [2004] (Fig. 3, constructs A, B, C, E, respectively).

HUMAN BMP2 REPORTER GENE CONSTRUCTS

All positions are indicated according to the distal start site of the human *BMP2* gene [Helvering et al., 2000].

BLuc (nucleotide (nt) −147 to 494, pGL2 h656x): A fragment containing −147 to 494 nt was generated by PCR using primers 5′-CGTCCACACCCCTGCGCGCAGCTCC-3′ and 5′-GGGCGCATTCT-CGAGCGAGTCGAGC-3′ which introduced a *Xho*I site in the PCR-generated fragment. This PCR fragment was phosphorylated using 10 units of polynucleotide kinase, digested with *Xho*I and then inserted into the *Sma*I and *Xho*I site of pGL2 basic vector (Promega, Madison, WI).

BLucB (pGL2 h656x HCNS): The mouse *BMP2* promoter sequence in the construct BmpLucH [Fritz et al., 2006] was excised by *Dra*III and *Xba*I digestion and replaced with a 1,357 nt *Dra*III and *Xba*I fragment containing the human promoter from the BLuc plasmid. The resulting plasmid contains a functional human *BMP2* promoter sequence (nt −147 to 494) and the human ultra-conserved *BMP2* 3′UTR sequence (nt 11,488–11,877).

BLucSV_B (pGL2 h656x pA HCNS): A *Hin*cII–*Hin*cII fragment of pGEM4 Human Koz-AccI [Fritz et al., 2004] containing the ultra-conserved *BMP2* 3′UTR sequence (nt 11,488–11,877) was inserted into the *Sal*I site of the BLuc construct described above.

CMVLUCBmp (pCβSLuchCNSHcAc) contains a *Hin*dIII–*Xba*I fragment containing the firefly luciferase-coding region from pGL3-basic (Promega) filled in with T4 DNA polymerase inserted into the *Sma*I site of pCβSGH [Natalizio et al., 2002] downstream of the CMV promoter. The human ultra-conserved sequence with the A rs15705 allele (nt 11,488–11,877) was inserted in a unique *Eco*RV site downstream of luciferase. This plasmid was converted to the C allele using the Stratagene QuikChange® II XL site-directed mutagenesis kit according to the manufacturer's instructions.

REPORTER GENE ASSAYS

Cells were plated in six-well plates (Costar, Corning, NY) and transfected with 500 ng luciferase reporter construct using FuGene6 Transfection Reagent (Roche, Indianapolis, IN) following the company's protocol. Twenty-four hours after transfection, cells were lysed with 1× Passive Lysis Buffer (Promega) and luciferase activities were measured using the Luciferase Assay System (Promega) following the company's protocol.

UV CROSS-LINKING

UV cross-linking was performed as described in Fritz et al. [2006]. Briefly, ^{32}P-labeled transcripts were incubated in cell extracts with or without unlabeled competitor RNA oligos in the presence of 1 mM EDTA for 10 min at 37°C. The protein–transcript complexes were cross-linked by ultraviolet light for 10 min using a 15-W germicidal lamp at 2 μJ/s at room temperature. After digestion of the unprotected RNA with 100 ng RNase A for 10 min at 37°C, cross-linked proteins were analyzed on 8% or 10% SDS–polyacrylamide gels and visualized and quantified using a Molecular Dynamics PhosphorImager and ImageQuant software.

UV CROSS-LINKING FOLLOWED BY IMMUNOPRECIPITATION

^{32}P-labeled RNA and protein complexes formed in the UV cross-linking reaction described above were incubated with an α-nucleolin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in immunoprecipitation buffer (50 mM Tris–Cl [pH 7.4], 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 with freshly added 1 mM PMSF and 0.2 mg/ml leupeptin) at 4°C for 1 h. Protein A/G-agarose beads (Amersham Pharmacia Biotech, Piscataway, NJ) were added and incubated overnight at 4°C with rotation. Beads were pelleted and extracted proteins were analyzed by 8% or 10% SDS–PAGE as described above.

RNA OLIGOS

All RNA oligos were synthesized by Dharmacon (Chicago, IL).

WESTERN BLOTTING

Total cell lysates were prepared in RIPA buffer (20 mM Tris 8.0, 137 mM NaCl, 10% [v/v] glycerol, 1% [v/v] NP-40, 0.1% [w/v] SDS, 0.5% Na-deoxycholate, 2 mM EDTA, pH 8.0, 1 mM PMSF, 1 mM aprotinin, and 20 μM leupeptin). One microgram of protein was separated by 10% SDS–PAGE and then transferred to nitrocellulose membrane at 18 V for 36 min in a semidry transfer apparatus (Bio-Rad Laboratories, Hercules, CA). The blots were incubated 1 h to overnight at 4°C with the specific primary antibody in 1× Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.5) with 1% (v/v) Tween-20 and 5% (w/v) non-fat dried milk. A polyclonal α-nucleolin antibody (Santa Cruz Biotechnology, Inc.) was used at a 1:1,000 dilution. Specific proteins were detected using BioWest Extended Duration Chemiluminescence Substrate (UVP, Inc., Upland, CA).

RNA AFFINITY CHROMATOGRAPHY

After pre-clearing with 40 μl streptavidin sepharose high performance beads (GE Healthcare Life Sciences, Uppsala, Sweden), 50 μl of cytoplasmic cell lysates [Fritz et al., 2004] was mixed with a 5′ end

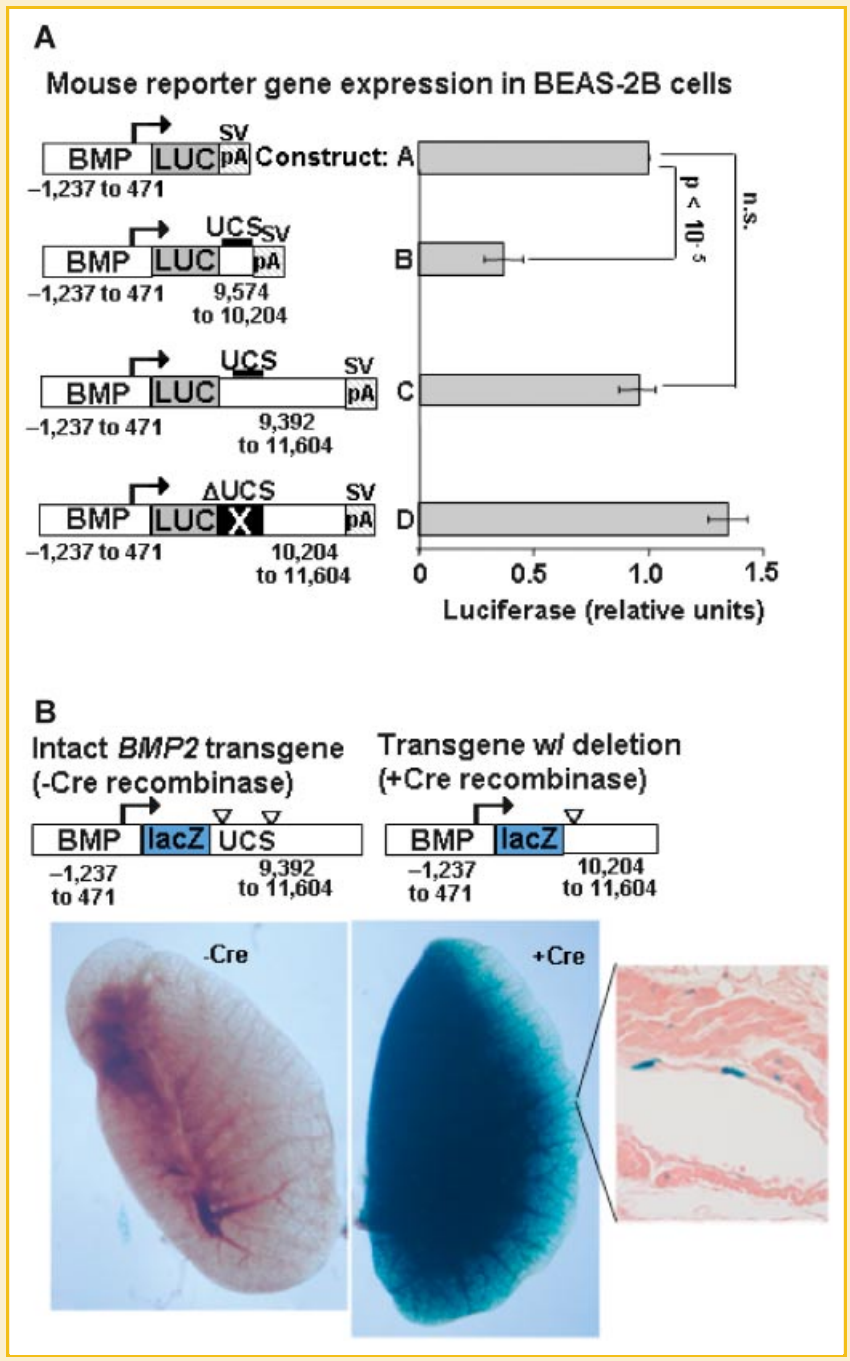

Fig. 1. The mouse ultra-conserved sequence (UCS) in the *BMP2* 3′UTR is a repressor in lung cells. A: Reporter constructs with the mouse *BMP2* distal promoter (nt −1,237 to 471) upstream of the luciferase (LUC) gene alone (construct A) or with nt 9,574–10,204 (construct B) or nt 9,392–11,604 (construct C) or nt 10,204–11,604 (construct D) of mouse *BMP2* sequence between the SV40 intron and the SV40 pA signal of pGL2-Basic. Average luciferase activity generated in non-transformed BEAS-2B cells after 24 h of transfection was normalized to total protein amount and graphed relative to the activity of the promoter-only plasmid (construct A) ± SEM, n = 4–6. A Student's *t*-test (two-sample assuming unequal variances) was used to compare the luciferase activity of the promoter-only plasmid (construct A) versus constructs containing the promoter and various portions of the *BMP2* 3′UTR and downstream flanking sequence (constructs B, C, and D). *P*-values larger than 0.05 are considered non-significantly different (n.s.), otherwise *P*-values are indicated. B: Diagrams of reporter transgenes with the murine *BMP2* promoter (, nt −1,237 to 471) and 3′UTR and downstream regions (nt 9,392–11,604) flanking the *lacZ* gene (−Cre). The *lacZ* gene encoded β-galactosidase fused with a nuclear localization signal (NLS). ▽ marks the loxP sites flanking the ultra-conserved sequence (UCS). To delete the ultra-conserved sequence in the transgene, mice carrying the *BMP2* transgene were mated with mice hemizygous for a ubiquitously expressed Cre-recombinase gene (+Cre). Lungs from day 2 pups were whole mount stained for β-gal activity (blue staining). Similar results were observed in adult lungs (not shown). A representative section of a β-gal stained lung from a mouse carrying the *BMP2* transgene with deletion of the ultra-conserved sequence (+Cre) demonstrates that the β-gal activity was restricted to the nucleus as expected.

biotin-labeled RNA containing the human *BMP2* sequence (nt 11,642–11,676) on ice in the presence of non-specific RNA (yeast tRNA). After proteins bound to the RNA at 30°C for 10 min, 40 μl of fresh streptavidin sepharose beads was added and incubated on ice for 30 min. Bound proteins were solubilized in 4× Laemmli buffer (250 mM Tris–HCl, pH 6.8, 40% [v/v] glycerol, 5% [p/v] SDS, 200 mM DTT, 0.005% [p/v] bromophenol blue) and separated by SDS–PAGE. The protein bands were visualized by Sypro Ruby staining. Proteins were excised from the gels, trypsin-digested, and analyzed by mass spectrometry.

PROTEOMIC ANALYSIS

Proteomic analysis was performed using an Applied Biosystems Voyager DE Pro matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrophotometer and a Micromass QTOF electrospray mass tandem spectrophotometer with advanced bioinformatics packages for protein identification by Dr. Hong Li's Lab in the Center for Advanced Proteomics Research at New Jersey Medical School [Wine et al., 2002].

RESULTS

AN ULTRA-CONSERVED BMP2 3′UTR SEQUENCE REPRESSED MOUSE BMP2 REPORTER GENES IN NORMAL LUNG CELLS IN VITRO AND IN VIVO

BMP2 is over-expressed in malignant lung tumors relative to benign tumors or normal lung tissues [Langenfeld et al., 2005a]. To understand this pathological expression, we investigated how the *BMP2* gene is regulated in lung cells. We previously reported that an ultra-conserved 3′UTR sequence of the murine *BMP2* gene regulates *BMP2* expression in F9 mouse embryonal carcinoma cells [Abrams et al., 2004; Fritz et al., 2004, 2006]. To test if the ultra-conserved sequence also regulates *BMP2* expression in tissue-cultured lung cells, we tested the effect of the ultra-conserved sequence in immortalized human bronchial epithelial cells (BEAS-2B) which do not express detectable *BMP2* mRNA and protein [Jiang et al., 2008]. BEAS-2B cells were transfected with luciferase reporter constructs containing the mouse *BMP2* distal promoter sequence and various subclones of the mouse *BMP2* 3′UTR and downstream sequence that had been previously analyzed in F9 cells [Fritz et al., 2004]. In contrast to the approximately threefold activating effect of the 630 nt ultra-conserved sequence in differentiated F9 cells that express *BMP2*, the ultra-conserved sequence strongly repressed the reporter gene by 68% in BEAS-2B cells (Fig. 1, compare constructs A and B). Deletion of the ultra-conserved sequence within the 2.2 kb sequence increased reporter gene activity (Fig. 1, compare constructs A and D). This is the first demonstration that the ultra-conserved sequence can repress *BMP2* expression in non-transformed BEAS-2B lung cells that do not express *BMP2*.

To test if the ultra-conserved sequence regulates *BMP2* expression in lung cells in vivo, we used transgenic mice which bear a transgene resembling construct C (Fig. 1A) with the mouse *BMP2* promoter region driving lacZ, the entire *BMP2* 3′UTR and 934 nt of downstream sequence [Fritz et al., 2006]. The 3′UTR was engineered to contain two loxP sites flanking the ultra-conserved sequence (Fig. 1B). The *BMP2* transgene with the intact 3′UTR was not expressed in normal lung cells (Fig. 1B, −Cre). This is consistent with reports indicating that BMP2 is barely detectable in normal lung tissues [Langenfeld et al., 2005a]. By mating these mice with the floxed reporter gene to Cre-recombinase-expressing mice [Schwenk et al., 1995], we generated transgenic mice with the ultra-conserved sequence deleted from the reporter transgene (Fig. 1B, +Cre). Deletion of the ultra-conserved sequence markedly induced reporter gene expression in fetal and adult (not shown) and post-natal lungs (Fig. 1B, $n > 20$). Thus, both in vitro and in vivo reporter gene data indicate that the ultra-conserved sequence inhibits *BMP2* expression in normal lung cells that do not express *BMP2*.

THE HUMAN ULTRA-CONSERVED SEQUENCE DIFFERENTIALLY REGULATED A HUMAN BMP2 REPORTER GENE IN NON-TRANSFORMED AND TRANSFORMED HUMAN LUNG CELLS

We have reported that, in contrast to non-transformed BEAS-2B cells, mycoplasma-transformed BEAS-2B cells and A549 lung adenocarcinoma cells synthesize high levels of *BMP2* [Jiang et al., 2008, Fig. 2A]. We hypothesized that the ultra-conserved sequence is bound by repressing factors in cells that do not make *BMP2* (e.g., normal lung cells) and activating factors in cells that do make BMP2 [e.g., transformed lung cells and differentiated F9 cells, Abrams et al., 2004; Fritz et al., 2004]. We tested this hypothesis by comparing the activity of reporter genes in the non-transformed or transformed BEAS-2B and A549 cells. We created a luciferase reporter construct (BLuc, Fig. 2B) that contains a functional human *BMP2* promoter driving a luciferase gene. Similar *BMP2* promoter sequences from primates are expressed appropriately in F9 cells [Abrams et al., 2004]. We compared the expression of BLuc to a plasmid with the same promoter, but with the human ultra-conserved sequence placed downstream of the luciferase gene (BLucB, Fig. 2B). BLuc and BLucB were transfected into non-transformed or transformed BEAS-2B cells and A549 cells. As previously demonstrated with the homologous mouse sequence (construct B, Fig. 1A), the human ultra-conserved sequence repressed BLucB expression by 62% in non-transformed BEAS-2B cells that do not express *BMP2* (Fig. 2C). Thus, the repressing function of the ultra-conserved sequence is conserved between human and mouse. In contrast, the ultra-conserved sequence stimulated BLucB expression by three- to fivefold in transformed BEAS-2B ($BEAS^t$) or A549 cells (Fig. 2C). Like differentiated F9 cells, transformed BEAS-2B and A549 cells express *BMP2* (Fig. 2A). These findings are the first demonstration that the ultra-conserved sequence differentially regulates *BMP2* expression in response to cell-specific factors and that these *trans*-regulating factors may up-regulate or down-regulate *BMP2* expression in lung cells.

THE ULTRA-CONSERVED SEQUENCE FUNCTIONS POST-TRANSCRIPTIONALLY

To test if the ultra-conserved sequence functions at the transcriptional level as a DNA element or at the post-transcriptional level as an RNA element, we measured luciferase RNA abundance in the cells transfected with each construct (BLuc or BLucB, Fig. 2D). If the ultra-conserved sequence regulates *BMP2* transcription, then luciferase RNA abundance should mirror luciferase activity. If

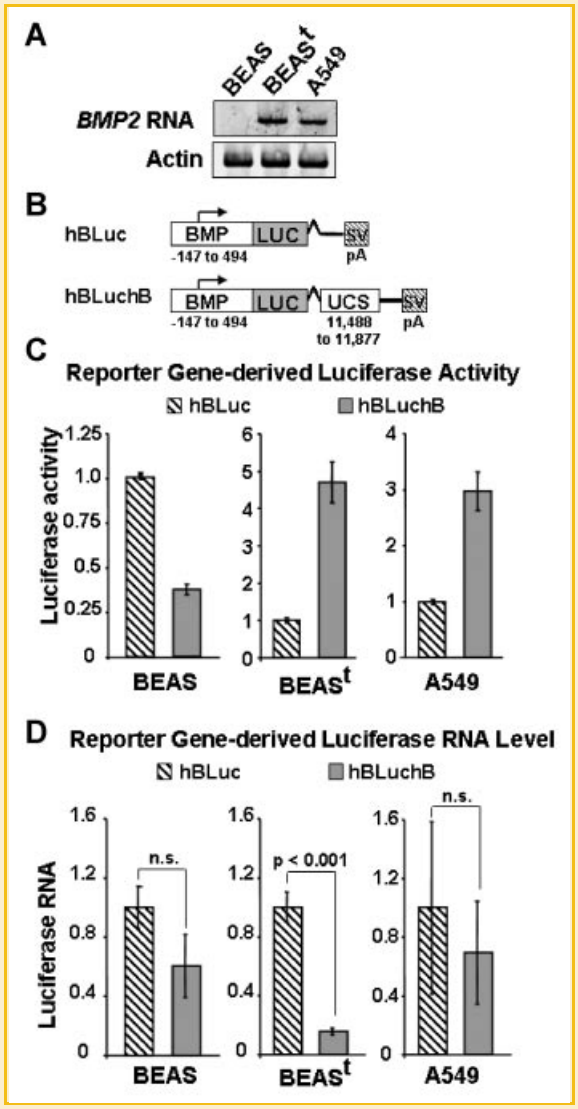

Fig. 2. An ultra-conserved sequence (UCS) in the *BMP2* 3′UTR is an activator in transformed human lung cells but is a repressor in non-transformed lung cells. A: RT-PCR using primers specific to the human *BMP2*. BMP2 mRNA was not detectable in normal BEAS-2B (BEAS) (immortalized bronchial epithelial cells) but was detected in transformed BEAS-2B (BEAS^t) and A549 (lung adenocarcinoma cells) ($n = 4$). β-Actin RT-PCR was used as a loading control. B: Diagrams of reporter constructs with the human *BMP2* promoter (\raster="-r="ueq001", nt −147 to 494 relative to the promoter) upstream of luciferase (BLuc) or the human *BMP2* promoter and the human ultra-conserved sequence (nt 11,488–11,877) between the SV40 intron (indicated by "∧") and the SV40 pA signal (SVpA) of pGL2-Basic (BLucB). C: Average luciferase activities generated in normal BEAS-2B (BEAS), transformed BEAS-2B (BEAS^t) or A549 cells 24 h after transfection were normalized to total protein amount and were graphed relative to the activity of the promoter-only plasmid (BLuc) ± SEM, $n = 3$–14. D: Average luciferase RNA levels were normalized to the amount of actin and graphed relative to the RNA level in cells transfected with the promoter-only plasmid (BLuc) ± SEM, $n = 2$–8. Statistical analysis was described in Figure 1.

post-transcriptional mechanisms are involved, then luciferase RNA abundance may differ from luciferase activity. In non-transformed BEAS-2B cells where the ultra-conserved sequence reduced luciferase activity by 62%, no significant reduction in RNA abundance was observed (Fig. 2D). The ultra-conserved sequence also did not significantly affect reporter RNA abundance in A549 cells, (Fig. 2D), although the ultra-conserved sequence increased luciferase activity by threefold. Interestingly, in the transformed BEAS-2B (BEAS^t) cells where the ultra-conserved sequence increased luciferase activity by fivefold (Fig. 2C), the ultra-conserved sequence decreased the reporter RNA abundance by 84% (Fig. 2D, compare construct BLuc vs. construct BlucB). This discordance between RNA abundance and luciferase activity may be because the luciferase RNA in those cells was being actively translated and thus was targeted to rapid RNA degradation [Aharon and Schneider, 1993; Curatola et al., 1995; Winstall et al., 1995]. These results support the hypothesis that the ultra-conserved sequence acts post-transcriptionally to regulate *BMP2* expression in lung cells.

To further test if the ultra-conserved sequence in the BLucB construct functions as a transcriptional enhancer in the plasmid DNA or a *cis*-regulatory element within the mRNA, we placed the ultra-conserved sequence downstream of the SV40 poly(A) signal in the pGL2 basic vector (Fig. 3A, construct BLucSV_B). Both BLucB and BLucSV_B contain the same DNA sequences. However, because the SV40 poly(A) signal is a strong viral poly(A) signal, the mRNA transcribed from the BLucSV_B construct should not contain the ultra-conserved sequence. To test if the SV40 poly(A) signal truncated the mRNAs efficiently, we performed RT-PCR to measure the presence or absence of the ultra-conserved sequence in the mRNA. The primer pairs were selected to span the SV40 intron of the pGL2-basic vector in order to distinguish cDNA-derived products from plasmid contamination. Primers A and B (vector-specific primers) were expected to produce RT-PCR products from RNAs generated from all constructs (BLuc, BLucB, BLucSV_B). In contrast, primers A and C (C is within the ultra-conserved sequence) can only generate products if the ultra-conserved sequence is in the RNA. Only the BLucB transcripts and any potential BLucSV_B transcripts that read through the SV40 poly(A) signal (depicted by a dotted line in Fig. 3A) would contain the ultra-conserved sequence. Primers A and B generated the expected RT-PCR products from cells transfected with all constructs (Fig. 3A). Using primers A and C, we only detected RT-PCR products generated from cells transfected with construct BLucB but not from construct BLucSV_B (Fig. 3A). Figure 3A shows data generated from transformed BEAS-2B. Similar results were observed in non-transformed BEAS-2B and A549 cells (data not shown). Because no transcripts extended through the SV40 poly(A) signal, the mRNAs transcribed from construct BLucSV_B were identical to the RNAs transcribed from construct BLuc that contains only the *BMP2* promoter.

We then compared the luciferase activity generated by these three constructs. As observed previously, the ultra-conserved sequence repressed reporter gene expression in non-transformed BEAS-2B cells but induced expression in transformed BEAS-2B cells and A549 cells (Fig. 3B). In contrast, the activity of the construct containing the ultra-conserved sequence placed downstream of the SV40 poly(A) signal (construct BLucSV_B) did not differ significantly from the activity of the construct containing only the *BMP2* promoter (construct BLuc) in all three cell lines (Fig. 3B). More importantly, the activity of the constructs containing the

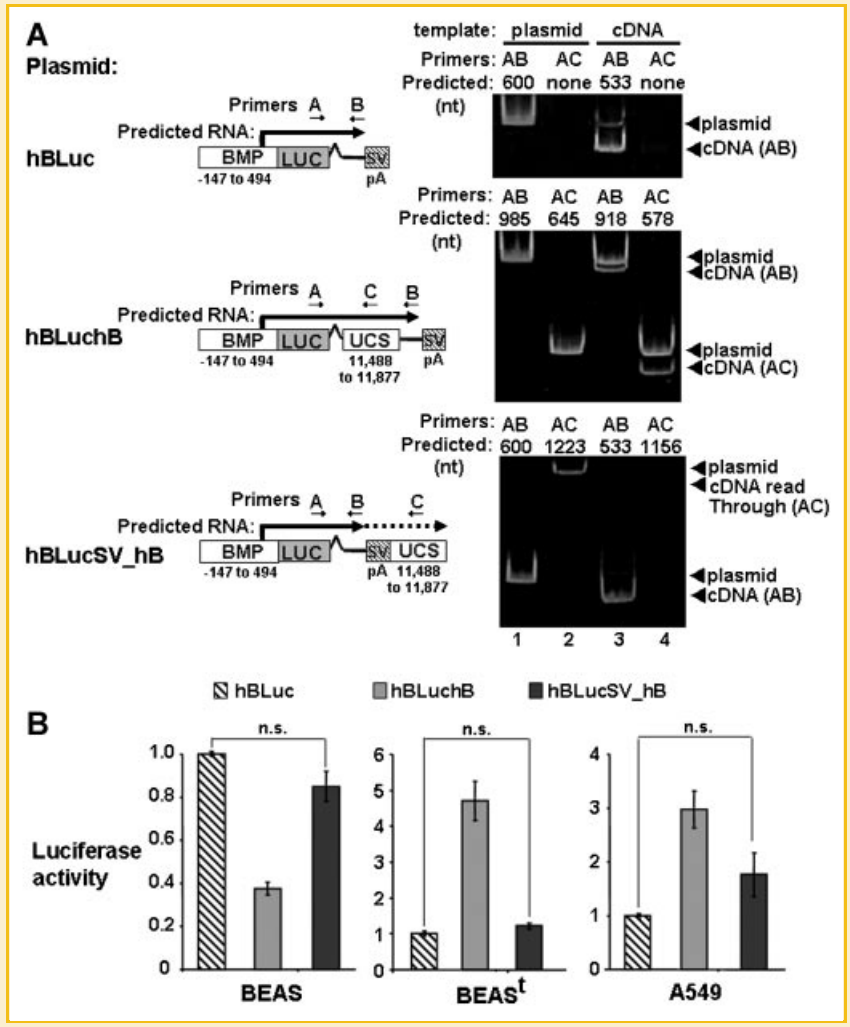

Fig. 3. The ultra-conserved sequence (UCS) in the *BMP2* 3′UTR regulates *BMP2* reporter gene expression at the post-transcriptional level. A: Left panel: diagrams illustrate the predicted length of the transcripts generated from reporter plasmids with the human *BMP2* promoter upstream of luciferase (BLuc) or with the promoter and the UCS immediately downstream of luciferase and the SV40 intron but upstream of the SV40 pA signal (SV) of pGL2-Basic (BLucB) or with same sequence inserted after the SV40 pA signal (BLucSV_B). In these diagrams, the 67 bp SV40 intron (not to scale) from the pGL2-Basic vector is indicated by "∧." PCR primers (A, B, C) flanking the intron were used to measure the RNA derived from each vector in transfected cells. Different PCR products (predicted size indicated in the right panel) were generated using different combinations of primers and different template DNA. Right panel: representative gels of PCR products. Lanes 1 and 2 were positive control PCR reactions with plasmids as template. Lanes 3 and 4 were PCR reactions using cDNA from RNA isolated from transfected transformed BEAS-2B cells. PCR products generated from plasmid DNA are 67 nt longer than that from cDNA because the plasmid DNA contains the intron sequence. Although the luciferase RNA was abundant in cells transfected with BLucSv_B as determined using the A and B primers, primers A and C failed to detect any 1,156 nt RNA that read through the SV40 pA signal. Likewise, no BLucSv_B RNAs that read through the SV40 pA signal were detected in normal BEAS-2B cells or A549 cells using the A and C primers (not shown). B: Average luciferase activities generated in normal BEAS-2B (BEAS), transformed BEAS-2B (BEAS^t) or A549 cells 24 h after transfection were normalized to total protein amount and graphed relative to the activity of the promoter-only plasmid (BLuc) ± SEM, n = 3–14. Statistical analysis was described in Figure 1. The luciferase activities generated from the BLuc and BLucSV_B plasmids were not significantly different (n.s.).

ultra-conserved sequence placed upstream (BLucB) or downstream (BLucSV_B) of the SV40 poly(A) signal differed significantly in all three cell lines (Fig. 3B). These data strongly support our hypotheses that the *BMP2* ultra-conserved sequence acts as a repressive mRNA element in cells that do not synthesize BMP2 and as an activating mRNA element in cells that do synthesize BMP2.

MAPPING PROTEINS THAT BIND TO THE ULTRA-CONSERVED SEQUENCE IN HUMAN LUNG CELLS

We previously noted that the ultra-conserved sequence in humans contains an A to C SNP (rs15705 SNP) which disrupts a conserved ARE [Fritz et al., 2006]. This polymorphism changed the interaction of specific proteins with the *BMP2* mRNA and the half-life of the *BMP2* RNAs in extracts from many cell types [Fritz et al., 2006; Devaney et al., 2009]. *Cis*-regulatory elements in the mRNA function by interacting with *trans*-acting factors to regulate essential post-transcriptional events including RNA processing, mRNA stability, and translation [Sengupta et al., 2004; Fahling et al., 2005; Takagi et al., 2005; Jiang et al., 2006; Zhang et al., 2006; Bunimov et al., 2007]. Cell-type-specific proteins from different tissues would differentially regulate BMP2 synthesis by binding regulatory elements in the *BMP2* mRNA. To understand the role of the

ultra-conserved region in lung cells, we began to identify the proteins that bind the ultra-conserved sequence. Because natural polymorphisms in the *BMP2* mRNA could change the affinities of proteins for the *BMP2* mRNA, we focused first on the proteins that bind to the rs15705 SNP region (Fig. 4A).

In vitro synthesized ^{32}P-labeled transcripts containing the full-length ultra-conserved sequence (nt 11,488–11,877, AF), or approximately the first half of the ultra-conserved sequence (nt 11,488–11,676, AL and CL) or a minimal 35 nt region (nt 11,642–11,676, AS and CS) centered on the rs15705 SNP at nt 11,659 were prepared (Fig. 4B). The full-length transcripts contained the A allele sequence. The two shorter RNAs were prepared with both the A and the C allele sequences. After UV cross-linking to cytoplasmic extracts from A549 cells and non-transformed or transformed BEAS-2B cells, proteins were separated by SDS–PAGE. This process yielded a profile of *BMP2* RNA binding proteins for each cell type, thus revealing both cell-specific and allele-specific differences. A pair of proteins that migrated at 110 kDa (p110) and 100 kDa (p100) were cross-linked by the full-length, long, and short 35 nt RNAs in A549 cells (Fig. 4), suggesting that they bound to the rs15705 SNP region of the *BMP2* mRNA. The *BMP2* RNAs cross-linked p110 in all three cell extracts, whereas p100 was apparent only in A549 and transformed BEAS-2B cell extracts. Thus, the sequence surrounding the rs15705 SNP binds cell-specific proteins.

THE rs15705 C ALLELE RNA HAS A HIGHER AFFINITY FOR THE p110 AND p100 PROTEINS THAN THE A ALLELE RNA

UV cross-linking demonstrated that p110 and p100 directly associated with a 35-nt *BMP2* RNA spanning the rs15705 SNP [Fritz et al., 2006]. We observed that the intensity of UV cross-linking between the A and C alleles and p110 and p100 in lung cell extracts differed. For example, p110 was labeled more strongly by the short C allele RNA than the A allele RNA in both non-transformed and transformed BEAS-2B cells. We hypothesized that these proteins bound to the A and C allele mRNAs with different affinities.

Because the direct UV cross-linking assay is not quantitative, we compared the binding affinity of the p110 and p100 proteins for the A or C allele RNAs using a quantitative RNA oligo competition for UV cross-linking assay [Fritz et al., 2006]. RNA oligo competition can interfere with RNA:protein interactions at a defined region. ^{32}P-labeled transcripts containing the full-length *BMP2* ultra-conserved sequence (A allele) were UV cross-linked with cell extracts in the presence of increasing amounts of cold 35 nt *BMP2* mRNA oligos whose sole difference was an A or C at the rs15705 SNP position. If the proteins UV cross-linked the full-length *BMP2* mRNA at the 35 nt region, then these RNA oligos should compete for binding and decrease cross-linking. Figure 5 shows that the A oligo inhibited cross-linking of p110 labeled by the full-length *BMP2* mRNA in

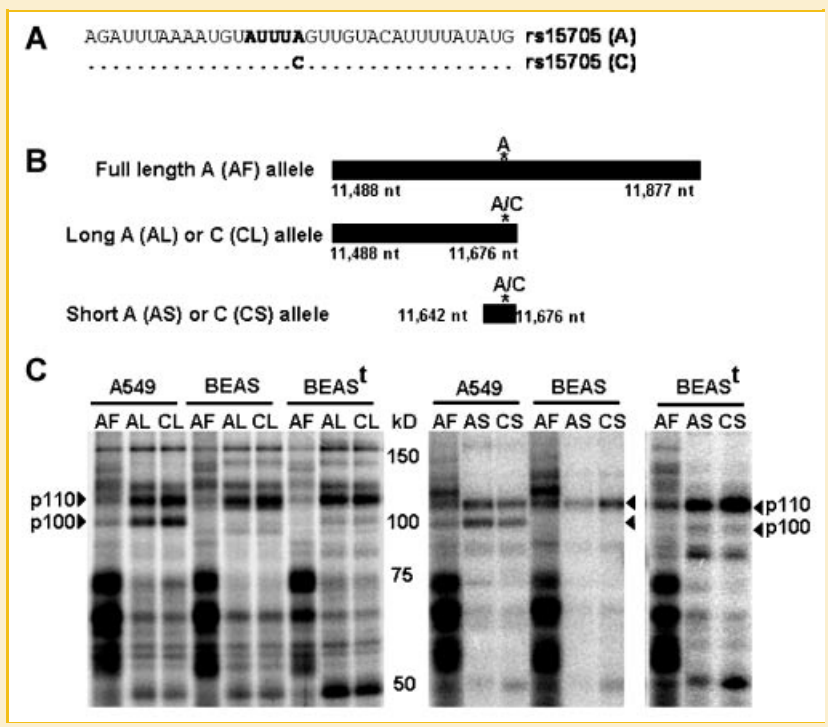

Fig. 4. Mapping proteins that bind near a SNP in the ultra-conserved sequence. A: The aligned sequences (nt 11,642–11,676 relative to the human distal promoter) show the A or the C allele at the rs15705 SNP. Unchanged nucleotides are indicated by "." B: Diagrams of RNAs used in UV cross-linking. *BMP2* RNA contained the full-length conserved *BMP2* sequence (nt 11,488–11,877) (AF), approximately the first-half of the conserved *BMP2* sequence (nt 11,488–11,676) (AL or CL) or 35 nt *BMP2* sequence (nt 11,642–11,676) (AS or CS). "*" indicates the location of the rs15705 SNP with the A allele in the AF, AL, or AS RNAs and the C allele in the CL or CS RNAs. C: ^{32}P-labeled *BMP2* RNAs were UV cross-linked in extracts from A549 cells or transformed BEAS-2B cells (BEAS^t), which both express *BMP2*, or normal BEAS-2B cells (BEAS), which do not express *BMP2*, with excess poly(A)competitor. A protein that migrates at about 110 kDa (p110) was labeled by the full-length and the shorter *BMP2* RNAs in all cell extracts used. A protein that migrates at about 100 kDa (p100) was strongly labeled in A549 extracts. p100 was weakly labeled in BEAS^t extracts, but not in BEAS extracts. Similar profiles were obtained in 2–8 independent experiments.

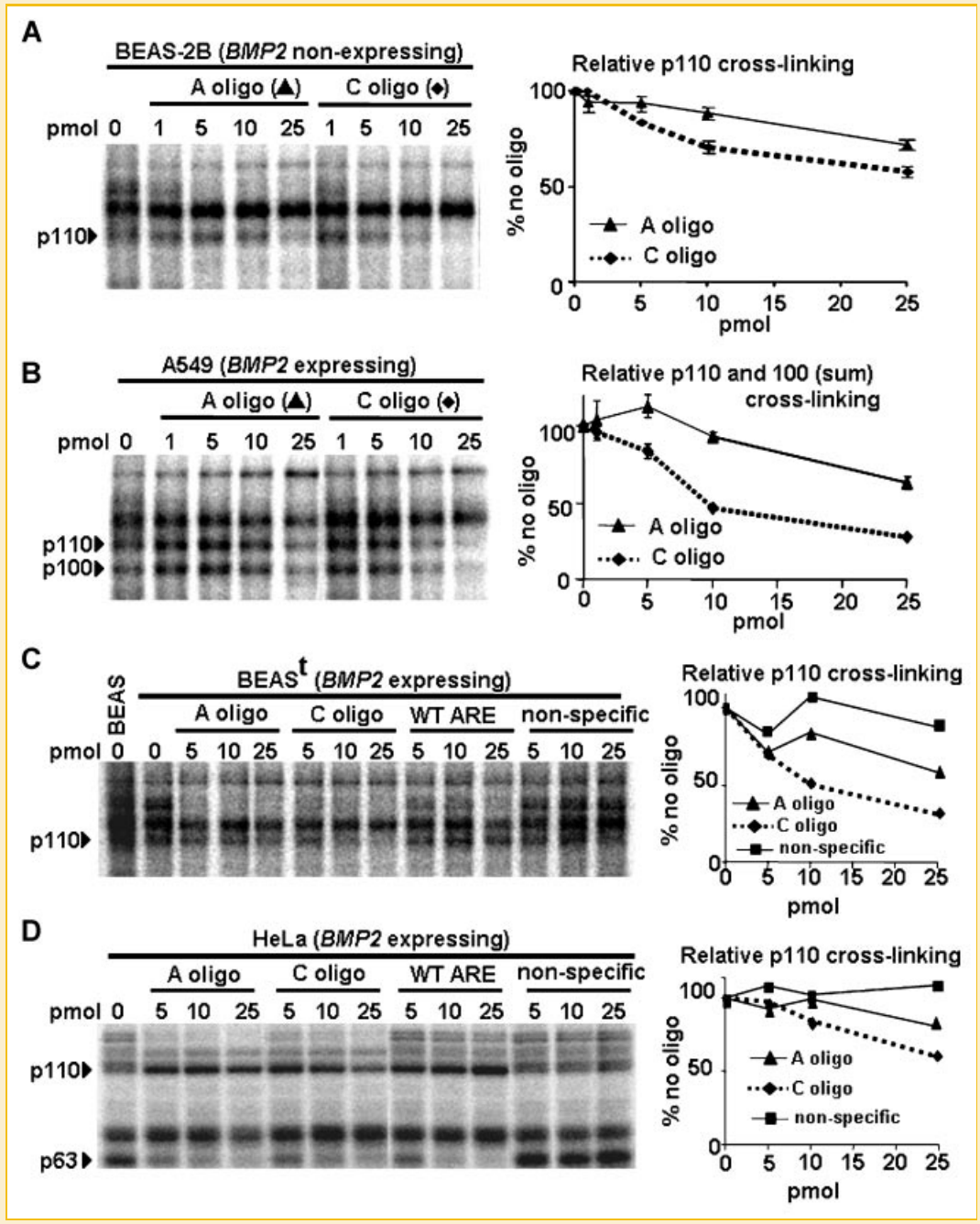

Fig. 5. The C allele *BMP2* RNA associates with the p110 and p100 more efficiently than the A allele *BMP2* RNA. ^{32}P-labeled human *BMP2* RNA with the full-length conserved *BMP2* sequence (nt 11,488–11,877) was UV cross-linked in extracts from normal BEAS-2B (BEAS), A549, transformed BEAS-2B cells (BEAS^t), or HeLa cells in the presence of excess poly(A) competitor and increasing amounts of RNA oligos as indicated. Representative gels are shown in the left panels. The average cross-linking to p110 or the sums of the p110 and p100 intensities relative to samples without oligo were graphed in the right panels ($n = 2$–3). A–D: *BMP2* RNA oligos (nt 11,642–11,676) with the A (A oligo, ▲, AGAUUUAAAAUGUAUUUAGUUGUACAUUUUAUAUG) or C (C oligo, ◆, AGAUUUAAAAUGUAUUUCGUUGUACAUUUUAUAUG) rs15705 allele sequence were used to compete. The A or C oligo sequences are the same as the AS or CS RNAs used in Figure 4. The C oligo competed more efficiently for a 110 kDa protein (p110) present in all cells tested. The C oligo also inhibited the cross-linking of an A549-specific 100 kDa protein (p100) more efficiently than the A oligo. C,D: RNA oligos bearing the wild-type TNF-α ARE (WT ARE, AAUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUUAA) or a non-specific RNA oligos (non-specific, GUCACGUGUCACC) were used to compete. The wild-type TNF-α ARE or the non-specific RNA oligos did not compete for p110 UV cross-linking whereas the *BMP2* RNA oligos did. D: In contrast, a protein that migrates at about 63 kDa (p63) was competed by wild-type TNF-α ARE RNA oligo but not by a non-specific RNA oligo.

non-transformed BEAS-2B, A549, transformed BEAS-2B, and HeLa cells. HeLa cell extracts were tested because HeLa cells also express *BMP2* [Jiang et al., 2008]. The A oligo also inhibited p100 cross-linking in A549 cells (Fig. 5B). These data confirmed that p110 and p100 bound to *BMP2* mRNA within the 35 nt surrounding the rs15705 SNP.

The A and C RNA oligos differ by only one nucleotide. If this single-nucleotide change alters protein affinities for this region, then the A and C oligo should compete for protein cross-linking differently. Figure 5 shows that the C RNA oligo inhibited p110 and p100 at a lower concentration than the A RNA oligo in all cell extracts tested. Thus, *BMP2* RNA bearing the C allele has a higher affinity for p110 and p100 relative to the *BMP2* RNA bearing the A allele.

The ultra-conserved region of the *BMP2* 3′UTR is highly AU-rich and contained several conserved AREs [Fritz et al., 2004]. To test if p110 binds to generally AU-rich sequences or a *BMP2* specific sequence, we used the classical and well-studied ARE from the tumor necrosis factor (TNF)-α mRNA to compete with the full-length *BMP2* mRNA in transformed BEAS-2B (Fig. 5C) or HeLa cell extracts (Fig. 5D). The classical ARE RNA contains multiple overlapping AUUUA motifs [Fritz et al., 2004]. A non-specific RNA oligo also was used to control for non-specific RNA binding. HeLa cells were used because extensive ARE-related information has been deduced from HeLa cell studies. Unlike the 35 nt BMP2 oligo, neither the TNF-α ARE oligo nor the non-specific, control RNA oligo blocked the cross-linking of the *BMP2* mRNA to the p110 protein in either transformed BEAS-2B cells or HeLa cells. In contrast, the ARE oligo, but not the non-specific, control oligo, competed efficiently for other proteins including proteins that migrated at approximately 63 kDa (p63) (Fig. 5D) and 32–33 kDa [Fritz et al., 2006]. These results indicate that the rs15705 C allele alters a *BMP2*-specific characteristic of the ultra-conserved sequence rather than simply disrupting a conserved AUUUA (ARE) sequence.

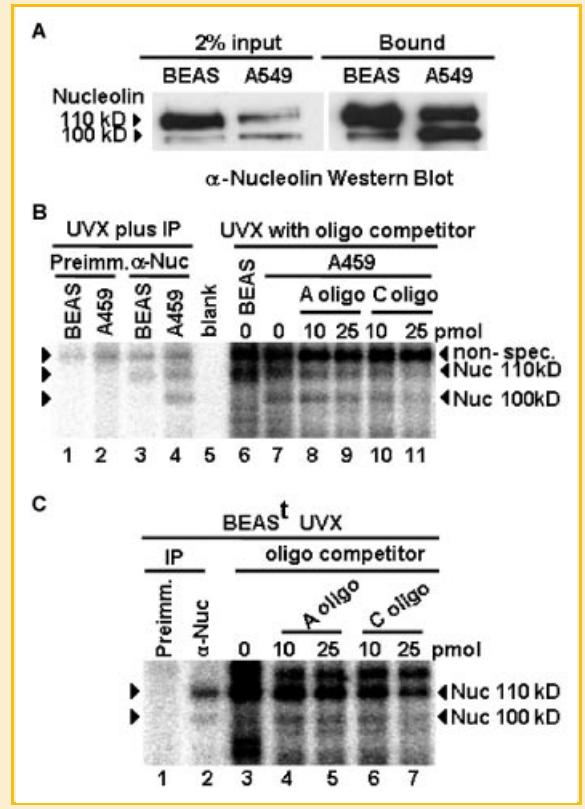

Fig. 6. Two forms of nucleolin bound to RNA with the *BMP2* conserved sequence. A: Normal BEAS-2B (BEAS) or A549 cell extracts were incubated with biotinylated *BMP2* RNA (nt 11,642–11,676) and streptavidin sepharose beads to pull down proteins that bind to BMP2 RNA. A Western blot using a polyclonal anti-nucleolin antibody shows that two forms of nucleolin that migrated at 100 and 110 kDa were captured by BMP2 RNA. B,C: A549, normal BEAS-2B (BEAS), or transformed BEAS-2B cells (BEAS^t) cell extracts were UV cross-linked (UVX) to the full-length ultra-conserved sequence followed by immunoprecipitation with anti-nucleolin (α-Nuc) antibody or pre-immune (Preimm.) IgG (lanes 1–4 in B, lanes 1–2 in C). Extracts were UV cross-linked (UVX) to the full-length ultra-conserved sequence in the presence or absence of the competitor RNA oligo (lanes 6–11 in B, lanes 3–7 in C) as described in Figure 5A and electrophoresed on the same gel to show that p110 and p100 are nucleolin (Nuc). Lane 5 in B was empty, n = 2.

p110 AND p100 ARE TWO FORMS OF NUCLEOLIN

To identify these proteins, we used biotin-labeled *BMP2* RNA containing the 35 nt conserved sequence (nt 11,642–11,676, Fig. 4A) to extract proteins from cytoplasmic extracts from non-transformed BEAS-2B or A549 cells. Proteomic analyses (see details in the Materials and Methods Section) indicated that nucleolin bound the *BMP2* mRNA. Nucleolin migrates in SDS–PAGE with an apparent molecular mass of 105 kDa [Srivastava and Pollard, 1999]. We pursued the effect of nucleolin binding because nucleolin has been shown to modulate RNA stability [Sengupta et al., 2004; Jiang et al., 2006; Zhang et al., 2006] and translation [Takagi et al., 2005; Bunimov et al., 2007] and is involved in oncogenic transformation [Takagi et al., 2005; Grinstein et al., 2006; Shi et al., 2007]. To confirm that nucleolin is bound to the *BMP2* mRNA, we performed Western blotting on the proteins that were extracted by the biotin-labeled *BMP2* mRNA using an anti-nucleolin antibody. Indeed, two forms of nucleolin, which migrated at approximately 110 and 100 kDa, were extracted by biotinylated *BMP2* RNA (Fig. 6A). As we observed by UV cross-linking (Fig. 5), non-transformed BEAS-2B cell extracts contained more 110 kDa nucleolin than the 100 kDa form, whereas A549 had equal amounts of 110 and 100 kDa nucleolin.

To prove that the p110 and p100 proteins that UV cross-linked to the *BMP2* RNAs (Fig. 5) are both nucleolin, we used an anti-nucleolin antibody to immunoprecipitate the UV cross-linked proteins. In non-transformed BEAS-2B cell extracts, p110 was labeled by the *BMP2* mRNA (Fig. 6B, lane 6) and was immunoprecipitated by the anti-nucleolin antibody (Fig. 6B, lane 3). In A549 (Fig. 6B, lanes 4 and 7–11) and transformed BEAS-2B cell extracts (Fig. 6C, lanes 2–7), both the p110 and p100 isoforms were immunoprecipitated. We electrophoresed the immunoprecipitated proteins on gels with total protein samples that were UV cross-linked to the *BMP2* RNA in the presence or absence of the competitor A or C oligos. Labeled proteins that were immunoprecipitated by the nucleolin antibody co-migrated with the same proteins that bound the competitor C oligo more efficiently relative

to the A oligo. Pre-immune rabbit IgG, a negative control, failed to precipitate any protein migrating at 110 or 100 kDa (Fig. 6B, lanes 1 and 2; Fig. 6C, lane 1). Thus, two forms of nucleolin bind the ultra-conserved sequence of *BMP2* RNA. Furthermore, the rs15705 C allele increases the affinity of this association.

THE rs15705 SNP IN THE ULTRA-CONSERVED SEQUENCE INFLUENCES BMP2 REPORTER GENE EXPRESSION IN LUNG CELLS

We compared two luciferase reporter genes driven by the constitutive cytomegalovirus (CMV) promoter and containing the entire human ultra-conserved sequence (CMVLUCBmp, nt 11,488–11,877) in non-transformed BEAS-2B cells and A549 adenocarcinoma cells (Fig. 7). These reporters differed only by an A or a C at the rs15705 SNP position. The constitutive CMV-driven reporter was used to eliminate any BMP2 promoter influence in different cell types. The reporter gene with the C allele was significantly more active relative to the identical plasmid with the A allele in BEAS-2B cells (Fig. 7; $P < 0.006$), but not in A549 cells. These results indicate that the rs15705 genotype in the BMP2 ultra-conserved sequence can influence BMP2 synthesis in lung cells.

DISCUSSION

Signaling molecules (e.g., growth factors) that mediate essential cellular events during embryonic development are tightly regulated in tissue- and stage-specific manners. Deregulation of these controls can lead to diseases including cancer. BMP2, but not other BMPs, is synthesized at significantly higher levels in malignant lung tumors relative to benign tumors or normal lung tissues [Langenfeld et al., 2005a]. Activating or repressing *trans*-acting factors are known to bind *cis*-acting DNA regulatory elements that mediate transcriptional gene up-regulation or down-regulation. We discovered that an ultra-conserved sequence of the *BMP2* mRNA is a potent regulatory switch that binds activating or repressing *trans*-acting factors to mediate post-transcriptional BMP2 gene regulation.

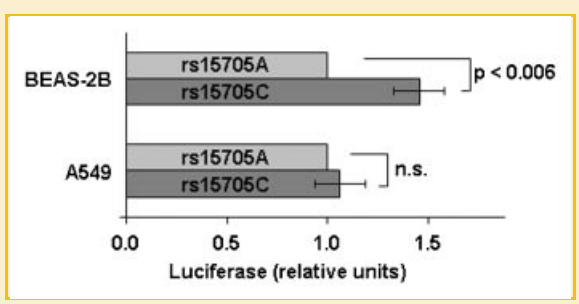

Fig. 7. The rs15705 genotype alters the luciferase activity generated from reporter genes with the ultra-conserved sequence. The A or the C rs15705 alleles in the human ultra-conserved sequence (nt 11,488–11,877) were inserted downstream of luciferase driven by the constitutively expressed CMV promoter in pCβSLuc [Devaney et al., 2009]. The local sequence is shown in Figure 4. The relative reporter activity ± SEM ($n = 10$) of each plasmid in A549 and non-transformed BEAS-2B cells is shown. Statistical analysis was described in Figure 1.

Consistent with our observations in differentiated F9 embryonal carcinoma cells that produce *BMP2* [Rogers et al., 1992; Rogers, 1996; Abrams et al., 2004; Fritz et al., 2004], the ultra-conserved sequence activated a *BMP2* reporter gene in two different types of BMP2-secreting human lung cells (A549 adenocarcinoma cells and transformed BEAS-2B immortalized bronchial epithelial cells) (Fig. 2). In distinct contrast, the ultra-conserved sequence repressed *BMP2* expression in non-transformed BEAS-2B immortalized bronchial epithelial cells (Figs. 1 and 2) that do not produce *BMP2*. Significantly, the repressive effect of the ultra-conserved sequence also occurs in normal mouse lung (Fig. 1B). These data show that the ultra-conserved sequence decreases *BMP2* expression in lung cells that do not express *BMP2*, while it stimulates *BMP2* expression in lung cells that do express *BMP2*. The 3′UTR serves as a platform for the combinatorial integration of activating or repressing factors. The specific combination of bound factors would either up-regulate or down-regulate BMP2 synthesis. Such a model would account for the highly dynamic and complex patterns of BMP2 synthesis that occur during development and in the adult organism. Elucidating the mechanisms, particularly in normal and malignant cells, may lead to novel treatment modalities.

In the absence of retinoic acid, undifferentiated F9 cells synthesize undetectable levels of *BMP2* mRNA [Rogers et al., 1992; Rogers, 1996]. We previously reported that retinoic acid increased *BMP2* transcription in F9 cells as measured by nuclear run-on assays [Heller et al., 1999]. Our subsequent studies showed that post-transcriptional mechanisms augment the potent ability of retinoic acid to induce *BMP2* expression in F9 cells [Fritz et al., 2004, 2006]. Recent studies in chondrocytes [Fukui et al., 2006] have shown that *BMP2* is initially transcriptionally up-regulated, followed by TNF-α-induced mRNA stabilization. We have proven here that the ultra-conserved sequence is a potent switch in the mRNA in normal and transformed lung cells. The ability of the 3′UTR to interact with either activators or repressors is a simple, but versatile, mechanism for modulating BMP2 synthesis in different cell types.

We have used proteomics and Western blot analysis of biotinylated BMP2 RNA affinity chromatography (Fig. 6) and immunoprecipitation of UV cross-linked BMP2 RNA (Fig. 6B,C) to establish that lung cell nucleolin binds the ultra-conserved *BMP2* 3′UTR sequence. Nucleolin is a multi-functional protein that shuttles between the nucleus, cytoplasm, and plasma membrane. In the cytoplasm, nucleolin can stabilize mRNAs [Sengupta et al., 2004; Jiang et al., 2006; Zhang et al., 2006] and inhibit translation [Takagi et al., 2005; Bunimov et al., 2007]. Nucleolin is over-expressed in many cancers and is involved in cellular transformation [Takagi et al., 2005; Grinstein et al., 2006; Shi et al., 2007]. We previously reported that a novel anti-cancer drug, AS1411 that binds to nucleolin [Girvan et al., 2006, #324], dramatically increased BMP2 synthesis in malignant lung cells [Jiang et al., 2008]. Over-expression of *BMP2* in lung cancer cells promotes lung tumor growth [Langenfeld et al., 2003, 2005b, 2006]. Understanding the relationship of nucleolin to BMP2 synthesis is relevant to lung cancer or other diseases where BMP2 synthesis is abnormal.

The common rs15705 SNP (22% C allele frequency) in the human *BMP2* 3′UTR [Styrkarsdottir et al., 2003] alters the affinity of the

BMP2 RNA for nucleolin and other proteins in vitro [Figs. 4–6, Fritz et al., 2006; Devaney et al., 2009]. At the molecular level, this would alter the subtle balance of post-transcriptional regulators on the BMP2 mRNA in cells. This would explain the differential reporter gene results observed in BEAS-2B cells (Fig. 7) and in other cell types [Devaney et al., 2009].

If similar effects occur in humans, then the precise level of *BMP2* may vary in individuals with the A or C genotype. We previously analyzed the effect of rs15705 on relative muscle, fat, and bone mass in humans, because myogenesis, adipogenesis, and osteogenesis have been established to be strongly influenced by BMP2 concentration. Homozygosity for the C rs15705 allele was associated with variation in levels of subcutaneous fat and whole arm and muscle volume before and after exercise [Devaney et al., 2009]. Our in vitro data and these population studies are consistent with the polymorphism causing changes in the precise level of BMP2 that lead to measurable human variation. Because increased *BMP2* mRNA levels are associated with poor lung cancer prognosis, it would be interesting to assess the distribution of the rs15705 alleles in lung cancer patients, as well as in other BMP2-associated pathologies such as atherosclerosis.

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