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Mycoplasma Infection Transforms Normal Lung Cells and Induces Bone Morphogenetic Protein 2 Expression by Post-Transcriptional Mechanisms

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Abstract Bone morphogenetic protein 2 (BMP2) is an essential growth factor and morphogen, whose pattern and level of expression profoundly influences development and physiology. We present the novel finding that mycoplasma infection induces *BMP2* RNA production in six cell lines of diverse types (mesenchymal, epithelial, and myeloid). Mycoplasma infection triggered the expression of mature secreted BMP2 protein in BEAS-2B cells (immortalized human bronchial epithelial cells), which normally do not express BMP2, and further increased BMP2 production in A549 cells (lung adenocarcinoma cells). Indeed, mycoplasma is as strong an experimental inducer as inflammatory cytokines and retinoic acid. Second, we showed that post-transcriptional mechanisms including regulation of RNA stability, rather than transcriptional mechanisms, contributed to the increased *BMP2* expression in mycoplasma-infected cells. Furthermore, a novel G-rich oligonucleotide, AS1411 that binds the post-transcriptional regulator nucleolin induced *BMP2* exclusively in infected cells. Finally, BMP2 stimulated proliferation in BEAS-2B cells transformed by chronic mycoplasma infection, as demonstrated by treatment with Noggin, a BMP2 antagonist. These findings have important implications regarding the effects of mycoplasma on BMP2-regulated processes, including cell proliferation, differentiation, and apoptosis. J. Cell. Biochem. 104: 580–594, 2008.

Key words: mycoplasma; bone morphogenetic protein 2; oncogenesis; transformation; lung cancer; post-transcriptional gene regulation

Mycoplasma, the smallest self-replicating prokaryote, is associated with various human diseases [Cimolai, 2001]. Mycoplasma infection can significantly affect the expression of many genes, including essential cytokines, growth factors, and oncogenes [Zhang et al., 2000, 2006a; Yang et al., 2002; Miller et al., 2003]. Mycoplasma infection may contribute to the pathogenesis and progression of human disease by causing the abnormal expression of key regulators of cell behavior. Chronic mycoplasma infection causes chromosomal changes [Fogh and Fogh, 1965; Paton et al., 1965; Tsai et al., 1995] associated with cell transformation and oncogenesis. Indeed, mycoplasma has been shown to cause malignant progression [Tsai et al., 1995; Feng et al., 1999].

Bone morphogenetic protein 2 (BMP2), an important member of the transforming growth factor-β (TGF-β) superfamily [Chen et al., 2004], regulates cell proliferation, apoptosis, differentiation, cell-fate determination, and morphogenesis [Chen et al., 2004]. Abnormal *BMP2* expression is associated with lung, breast, colon, prostate, and pancreatic cancers [Harris et al., 1994; Reinholz et al., 2002; Langenfeld et al., 2003; Hardwick et al., 2004;

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Langenfeld et al., 2005]. The roles of BMP2 in tumorigenesis vary in a tissue-specific and/or tumor-type-specific manner [Kleeff et al., 1999; Ghosh-Choudhury et al., 2000a; Ghosh-Choudhury et al., 2000b; Reinholz et al., 2002; Langenfeld et al., 2003; Hardwick et al., 2004; Horvath et al., 2004; Langenfeld et al., 2005; Raida et al., 2005]. BMP2 may act as a tumor suppressor in prostate, breast, and colon cancer [Soda et al., 1998]. In lung, BMP2 synthesis is significantly increased in all types of lung carcinomas compared to normal tissues or benign lung tumors [Langenfeld et al., 2005]. Elevated BMP2 levels in lung tumors enhance tumor growth by increasing cell proliferation and migration, and by stimulating angiogenesis [Langenfeld et al., 2003; Langenfeld and Langenfeld, 2004; Langenfeld et al., 2006]. Furthermore, studies in two mouse models have shown that the BMP2 antagonist Noggin reduces metastasis to lungs and bones [Feeley et al., 2006; Langenfeld et al., 2006]. However, the mechanisms of how BMP2 synthesis is activated in malignant lung cells remain unknown. The incidence of mycoplasma infection was reported to be significantly higher in small cell lung carcinoma patients than in a healthy control group [Pehlivan et al., 2004]. Also, we have found that 11 out of 11 samples from lung cancer patients were mycoplasma positive. An influence of mycoplasma on *BMP2* expression could influence the onset and progression of lung cancer and other human diseases.

We discovered that mycoplasma infection induces BMP2. This observation is relevant to many areas of cell biology. First, mycoplasma infection may influence lung cancer progression. Second, extensive effort is being devoted to identifying cell- and virus-based systems to deliver BMP2 for orthopedic uses. Undetected mycoplasma contamination would completely change the results of these studies. Third, BMP2 is a classic morphogen whose potent influence on a wide variety of cells from the embryo to the adult changes with concentration. Low-level mycoplasma contamination would exert significant effects on a host of basic cell biological results. Indeed, depending on the survey, 15–80% of cell cultures are mycoplasma-contaminated [Fleckenstein et al., 1994; Uphoff and Drexler, 2002]. Finally, our observations add to a growing body of data indicating that post-transcriptional processes are an essential aspect of *BMP2* gene regulation.

MATERIALS AND METHODS

Materials

Recombinant human Noggin was a gift from Aris Economides or purchased from R&D system (Minneapolis, MN). The G-rich oligonucleotide AS1411 (GGTGGTGGTGGTTGTGGTGGTGGTGG) or inactive GRO (TTGGGGGGGGTGGGT) or CRO (CCTCCTCCTCCTTCTCCTCCTCCTCC) oligos were synthesized by Integrated DNA Technologies (Coralville, IA). Tissue culture and molecular reagents were obtained from Sigma Aldrich (St. Louis, MO) and Invitrogen (Carlsbad, CA).

Cell Culture

HeLa, MCF-7, A549, and BEAS-2B cells were cultured in DMEM (Sigma Aldrich, St. Louis, MO) with 5% fetal bovine serum and 1% glutamine. 32D cells were maintained in RPMI culture medium containing 15% fetal bovine serum and 5% WEHI-3B-conditioned medium. The $C_3H/10T_{1/2}$ (clone 8) cell line (ATCC CCL 226) was grown in RPMI 1640 medium supplemented with 5% fetal bovine serum (Invitrogen, Carlsbad, CA). All cells were grown in 5% CO_2 at 37°C.

Mycoplasma Infection

Unintentionally mycoplasma-infected HeLa, MCF-7, and A549 cells were obtained from other labs. Uninfected BEAS-2B and A549 cells were intentionally infected using conditioned-media from mycoplasma-infected A549 cells as follows. Mycoplasma-free cells were seeded in 100 mm plates (Falcon, Franklin Lakes, NJ) with 5 ml complete media and 5 ml conditioned-media from mycoplasma-infected cells. After 2 days, media was removed, cells were washed with PBS, and then cultured in fresh complete media. Infected and control uninfected cells were confirmed to be mycoplasma-positive or -negative, respectively, using the "MycoAlert Mycoplasma Detection Kit" (Cambrex, Rockland, ME). Control uninfected cells were cultured and harvested in parallel.

Identification of Mycoplasma Species

Mycoplasma rDNA (16S) was PCR amplified from DNA extracted from the conditioned medium (CM) of infected cells. The primer set used for PCR detected the highly conserved

mycoplasmal rDNA sequences was 5′-ACTCC-TACGGGAGGCAGCAGTA-3′ and 5′-TGCAC-CATCTGTCACTCTGTTAAC-3′. The amplified DNAs were then sequenced. BLAST analysis revealed that the sequences closely matched the *Mycoplasma arginini* 16S rDNA sequence.

Infection of 32D Cells With Defined Mycoplasma Species

32D cells were transferred to IL-3 free culture medium, and then inoculated with *M. fermentans* (incognitos strain) and *M. hominis* at a ratio of 1,000 color change units/cell as previously described [Feng et al., 1999]. *M. fermentans* and *M. hominis* were grown in SP-4 medium and titered before inoculation into cell cultures.

Eradication of Mycoplasmas in Cell Cultures

To eradicate mycoplasmas from the infected 32D cell cultures, the cultures were treated with ciprofloxacin (10 mg/ml) for 3–4 weeks and confirmed to be mycoplasma-negative as previously described [Feng et al., 1999].

RNA Isolation

Total RNAs from mycoplasma-positive or -negative HeLa, MCF-7, A549, and BEAS-2B cells were isolated using the guanidinium isothiocyanate cesium gradient method [Ausubel et al., 1997]. Total RNAs from 32D and $C_3H/10T\frac{1}{2}$ cells were isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Northern Blot

Total RNA (30 μg) isolated from mycoplasma-infected or non-infected cells was analyzed as described [Hosler et al., 1993]. The human *BMP2*-specific cDNA probe was a *Pvu II-Xba I* gel-purified fragment from an EST clone (*GenBank* accession no. AA113794). The probe contains from −1,259 nt to −426 nt upstream of the stop codon. Hybridized bands were visualized and quantified using a Molecular Dynamics PhosphorImager.

RT-PCR

Total RNA (1 μg) from mycoplasma-infected or non-infected cells was reverse transcribed using 200 units of SuperScript™ III RT (Invitrogen, Carlsbad, CA) and 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 first intron (exon 1 primer 5′ CTTGGCTGGAGACTTCTTGAAC 3′, exon 2 primer 5′ CCAGGAGGACCTGGGGAAGCAG-CAA 3′). The predicted size of the amplified cDNA fragment is 423 bp, whereas a product generated from genomic DNA would be 1,630 bp. For BMP4 PCR, primers spanned the fourth intron (exon 4 primer 5′ ACCTGAGACGGGG-AAGAAAA 3′, exon 5 primer 5′ TTAAAGAGG-AAACGAAAAGCA 3′). The predicted size of the amplified cDNA fragment is 348 bp, whereas a product generated from genomic DNA would be 1,313 bp. The conditions for the BMP2 and BMP4 reactions were: 95°C, 2 min; followed by 30 cycles of 94°C, 1 min; 55°C, 30 sec; 72°C, 1 min; ending with 72°C, 7 min. For β-actin PCR, primers spanned the second intron (exon 2 primer 5′ CGTGGGGCGCCCCAGGCACCA 3′, exon 3 primer 5′ TTGGCCTTAGGGTTCAGGG-GGG 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, 2 min; followed by 30 cycles of 95°C, 1 min; 60°C, 1 min; 72°C, 1 min; ending with 72°C, 7 min.

ELISA

Uninfected or mycoplasma-infected A549 or BEAS-2B cells were cultured in a 100 mm dish with 7 ml complete media. After 4 days, CM was collected and pre-cleared by centrifugation of 2 min at 4,000*g* at 4°C to remove dead cells. Precleared CM (2 ml) was concentrated using a Centricon Centrifugal Filter Unit with Ultracel YM-10 membrane (Millipore, Bedford, MA). The Quantikine BMP2 Immunoassay ELISA kit (R&D system, Minneapolis, MN) was used to quantify the BMP2 protein level in 50 μl concentrated CM.

Reporter Gene Assays

A PCR-generated fragment containing the human *BMP2* distal promoter (from nt −147 to 494 relative to the start site) was cloned into the Sma I and Xho I site of the pGL2 basic vector (Promega, Madison, WI). A plasmid containing human *BMP2* promoter sequence (2185p1-2 5.5 EcoRI/Fsp(p) huBmp2pGL3) provided by Dr. S. Harris [Feng et al., 1997] was used as templete. Uninfected or mycoplasma-infected BEAS-2B cells were plated in 6-well plates (Costar, Corning, NY) and tranfected with 500 ng

luciferases reporter construct using FuGene6 Transfection reagent (Roche, Indianapolis, IN) following the company protocol. Twenty-four hours after transfection, cells were lysed with 1X Passive Lysis Buffer (Promega, Madison, WI) and luciferase activities were measured using the luciferase assay system (Promega, Madison, WI).

In Vitro RNA Stability Measurements

The in vitro decay rates of capped and ^{32}P-labeled BMP2 RNAs in S18 cytoplasmic extracts were quantified as in Fritz et al. [2004b].

UV Cross-Linking

^{32}P-labeled transcripts were cross-linked by ultraviolet light in cell extracts with 1 mM EDTA as in Fritz et al. [2006].

Cell Morphology Observation

Uninfected or mycoplasma-infected BEAS-2B cells were plated at 530 cells/cm^2. After 5 days culture, the morphology of the cells was observed at 100× magnification by phase contrast (Nikon ECLIPSE TE300, Japan). Images were captured using Spot Advanced Software (Diagnostic Instruments, Sterling Heights, MI).

Cell Proliferation Assay

To determine cell growth rate, 2.6×10^4 cells were plated on 12-well plates (Costar, Corning, NY). Live, trypan blue-excluding cells were counted with a hemocytometer.

Colony Formation in Soft Agar

Colony formation in soft agar was performed as described with slight modification [Korah et al., 2000]. Cells were seeded in a 0.3% Bacto Agar (Difco Laboratories, Detroit, MI) overlay on a preformed 0.6% agar layer (0.667 × DMEM, 6.7% fetal bovine serum). Plates (35 mm) containing 1,000, 2,000, 4,000, and 8,000 cells (each in triplicate) in agar were incubated at 37°C in 5% CO_2. Colony formation was observed under a phase contrast inverted microscope with 40× magnification at 10 days. Colony images were captured using Spot Advanced Software (Diagnostic Instruments, Sterling Heights, MI). Colonies also were stained with 0.17 mg/ml neutral red overnight and photographed as whole plates using a Syngene Bio Imaging system (Syngene, Frederick, MD).

Tumor Growth in Nude Mice

Ten million (10^7) cells were injected subcutaneously into female NCJ athymic nude mice. Eight weeks following injection the animals were sacrificed and the tumors were removed and measured in three dimensions. These studies were approved by the Robert Wood Johnson Medical School Institutional Animal Care and Use Committee.

RESULTS

Mycoplasma Infection Induced *BMP2* RNA Expression in Four Human Cell Lines

We conducted a survey of *BMP2* expression in different cell types. Using Northern blots with a *BMP2*-specific probe, we detected a full length expected *BMP2* RNA (*GenBank* accession no. NM_001200) and a shorter novel transcript in several cell lines (Fig. 1A). Curiously, different isolates of the same cell lines had different *BMP2* RNA patterns. For example, A549 cells (lung adenocarcinoma cells) from different labs (A549-A, A549-B, and A549-C, Fig. 1A) expressed different levels of *BMP2*. A549-A and A549-B cells expressed undetectable or low levels of the full length *BMP2* RNA, respectively, while A549-C expressed a high level of both the expected *BMP2* and the novel RNAs (Fig. 1A, lanes 1, 3, 5). Similarly, two sublines of breast cancer cells MCF-7 (A, B) expressed *BMP2* differently (Fig. 1A, compare lanes 6, 7). Finally, two sublines of HeLa cells (A, B) both expressed the full length *BMP2* RNA, but only HeLa-B expressed the shorter RNA (Fig. 1A, compare lanes 8, 9). We subsequently discovered that the high level of *BMP2* RNA expression and the novel transcript correlated with occult *Mycoplasma arginini* infection (Fig. 1A).

We hypothesized that mycoplasma infection induces *BMP2* expression in these cells. To test this hypothesis, we intentionally exposed non-*BMP2* expressing cells, A549-A, A549-B, and BEAS-2B (immortalized human bronchial cells) to conditioned-media from mycoplasma-positive A549-C cells for one passage. Low level or no *BMP2* RNA was detected in the uninfected A549-A, A549-B, or BEAS-2B cells (Fig. 1A, lanes 1, 3, 10). In contrast, both the full length *BMP2* and the novel RNAs were strongly induced in the mycoplasma-infected cells relative to the uninfected control cells (Fig. 1A, lanes 2, 4, 11). This data is consistent with the

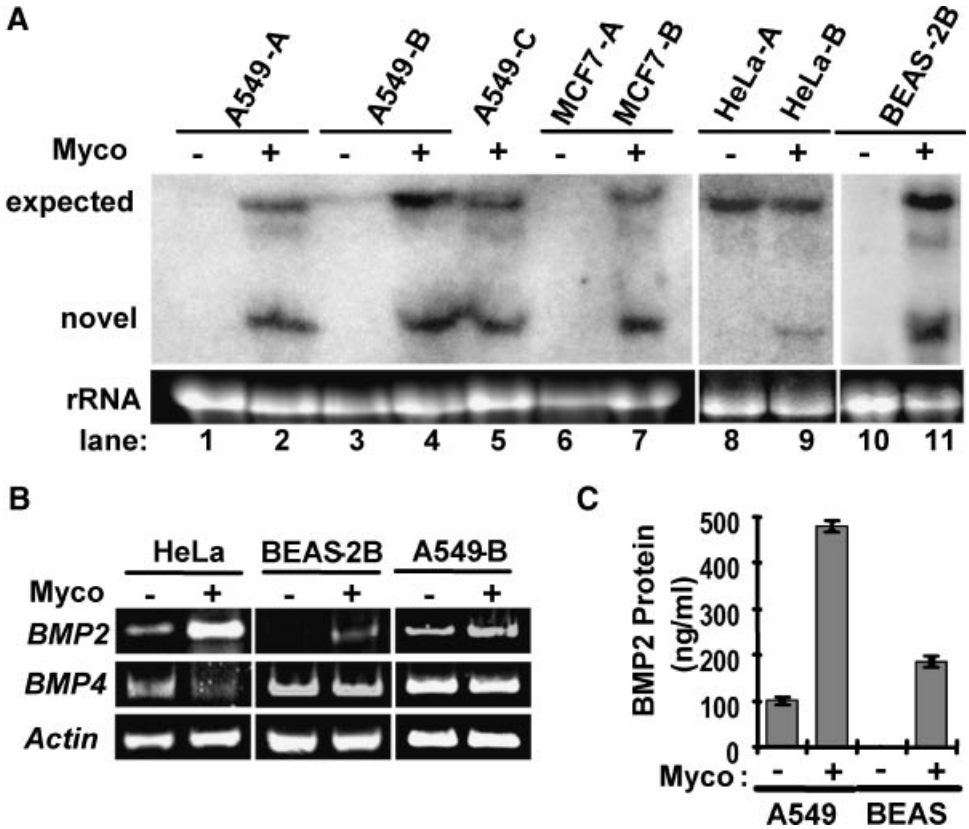

Fig. 1. Mycoplasma infection induces *BMP2* RNA and protein in human cell lines. **A**: Northern blot of *BMP2* mRNA from mycoplasma positive or negative cells. A549-C, MCF7-B, HeLa-B were infected through contamination prior to the acquisition of the cells. Mycoplasma-positive A549-A (**lane 2**), A549-B (**lane 4**), BEAS-2B (**lane 11**) were intentionally infected by adding CM from mycoplasma positive (*M. arginini*) A549-C cells for one passage. Ethidium bromide stained rRNA was used as a loading control. **B**: RT-PCR using primers specific to the human *BMP2* or *BMP4* gene. Consistent with the Northern blot results, *BMP2* mRNA levels are increased in the infected cells. In contrast, mycoplasma-positive or -negative cells BEAS-2B and A549 cells expressed equal levels of *BMP4* mRNA. *BMP4* mRNA level decreased in mycoplasma-infected HeLa cells. β-actin RT-PCR was used as a loading control. **C**: Uninfected (**Myco**−) or mycoplasma-infected (**Myco**+) A549-B (**A549**) or BEAS-2B (**BEAS**) cells were cultured for 4 days. BMP2 protein levels in the concentrated CM were detected by ELISA (n = 3). Mature, secreted BMP2 protein levels were increased in mycoplasma-infected cells relative to uninfected cells.

hypothesis that *BMP2* expression is induced by mycoplasma infection.

Some uninfected cells expressed the normal expected *BMP2* RNA, for example, HeLa cells and the B subline of the A549 cells. In contrast, all mycoplasma-infected cells also expressed the low molecular weight transcript. The *BMP2* and *BMP4* amino acid sequences are highly similar and the *BMP4* mRNA is smaller than the *BMP2* mRNA. Although we have never observed cross hybridization of the mouse *BMP2* and *BMP4* probes, we considered the formal possibility that the shorter transcript was *BMP4*. The human *BMP2* probe shares only 4.4% identity over 833 nt to the human *BMP4* mRNA; however, the nucleotide sequences are more similar over short stretches of sequence. To eliminate potential cross hybridization, we perform semi-quantitative RT-PCR using primer pairs specific to the human *BMP2* and *BMP4* sequences on RNA from mycoplasma-infected or -uninfected HeLa, BEAS-2B, and A549-B cells. In contrast to *BMP2*, mycoplasma-infection did not induce the *BMP4* RNA level in these cells (Fig. 1B). Indeed, mycoplasma-infection reduced the abundance of the *BMP4* RNA in HeLa cells. Furthermore, the BMP2 probe failed to hybridize to any RNA in uninfected BEAS-2B cells (Fig. 1A, lane 10) although the RT-PCR indicated that the *BMP4* RNA is present in uninfected BEAS-2B cells. Thus, the probe used for the Northern blot was *BMP2* specific. Similar results were observed using cDNAs generated by oligo dT or random hexamer-primed reverse transcription. These results indicate that the novel transcript

induced by mycoplasma infection is a variant of *BMP2*.

We then inspected the UniGene database for known *BMP2* transcript variants. A 3,150 bp *BMP2* mRNA sequence (*GenBank* accession no. NM_001200) and a 1,547 bp *BMP2* mRNA sequence (*GenBank* accession no. M22489) closely represent the full length and shorter transcripts found in mycoplasma-infected cells. Alignment of these sequences showed that the 1,547 bp sequence was truncated at the 5′ and 3′ ends relative to the 3,150 bp sequence.

Mycoplasma Infection Induced Mature BMP2 Protein in Cell Culture Medium

We then examined whether mycoplasma infection induced *BMP2* expression at the protein level. Uninfected control or mycoplasma-infected A549-B and BEAS-2B cells were cultured for 4 days. The mature secreted form of BMP2 protein in the cell culture medium was measured by ELISA. Mycoplasma infection significantly induced the BMP2 protein level in both A549-B and BEAS-2B cells (Fig. 1C). In infected A549-B cells, the BMP2 level was nearly five times that of the uninfected cells. The BMP2 protein was undetectable in the uninfected BEAS-2B cells, but was strongly induced following infection. Thus, BMP2 protein levels are induced by mycoplasma infection.

M. fermentans and *M. hominis* Rapidly Induced *BMP2* Expression in Mouse Myeloid Cells

The effects of mycoplasma infection on gene expression can vary with host cell-type, species of infecting mycoplasma, and length of infection [Zhang et al., 2000]. The 32D mouse myeloid cell line is IL-3-dependent. Removal of IL-3 from cell culture leads to rapid apoptosis. In 32D cells, some mycoplasma species (e.g., *M. hominis*) promote apoptosis while some species (e.g., *M. fermentans*) rescue IL-3-deprived cells from undergoing apoptosis [Feng et al., 1999]. Because *BMP2* causes apoptosis in many cell types [Glozak and Rogers, 1996; Hay et al., 2001; Hallahan et al., 2003; Zhang et al., 2003], we hypothesized that apoptosis-promoting mycoplasmas, but not apoptosis-preventing mycoplasmas, induced *BMP2* in 32D cells. We infected 32D cells with *M. fermentans* and *M. hominis* for 4, 12, 24, and 36 h in IL-3-free culture. Uninfected control cells did not express *BMP2* (Fig. 2A). In contrast, both *M. fermentans* and *M. hominis* induced similar levels of the expected and novel *BMP2* mRNAs within 4 h of infection (Fig. 2A). The similar abilities of both *M. fermentans* and *M. hominis* to rapidly induce *BMP2* suggest that differences in *BMP2* induction do not explain the apoptosis-promoting effect of *M. hominis*.

M. fermentans and *M. penetrans* Induced *BMP2* Expression in Mouse Fibroblasts

Chronic infections with either *M. fermentans* or *M. penetrans* have been shown to promote reversible (mycoplasma presence necessary) and irreversible (mycoplasma presence unnecessary) transformation of the murine embryonic $C_3H/10T_{1/2}$ (C_3H) cell line [Tsai et al., 1995]. Because elevated *BMP2* levels are associated with oncogenesis in some cells [Langenfeld et al., 2003, 2005; Langenfeld and Langenfeld, 2004], we measured the level of *BMP2* expression during different stages of mycoplasma-mediated transformation (Fig. 2B). C_3H cells were infected with *M. fermentans* or *M. penetrans* for 1 week, 7 weeks, 11 weeks, and 18 weeks. These time points reflect different stages of mycoplasma-induced malignant transformation [Tsai et al., 1995]. In the control uninfected C_3H cells, *BMP2* RNA was undetectable. After 1 week of infection by either *M. fermentans or M. penetrans*, but prior to transformation, the *BMP2* RNA was slightly induced. In contrast, both the expected and the novel *BMP2* mRNAs were significantly induced in the reversible stage (7 or 11 weeks infection by either species) of transformation. BMP2 continued to be expressed strongly in cells that were irreversibly transformed by *M. fermentans* (18 weeks infection). In contrast, cells irreversibly transformed by *M. penetrans* expressed lower levels of BMP2. This is consistent with the hypothesis that *BMP2* promotes the onset of transformation, but may not be required to maintain transformation.

Because the C_3H cells expressing *BMP2* were both mycoplasma-infected and transformed, we could not distinguish whether mycoplasma induces *BMP2* or transformation induces *BMP2*. We tested this by measuring *BMP2* expression in *M. fermentans*-transformed 32D cells cured by ciprofloxacin treatment. After transformation by *M. fermentans*, the strictly IL-3-dependent 32D cells grew rapidly in IL-3-free culture [Feng et al., 1999]. *BMP2* RNA was not detected when

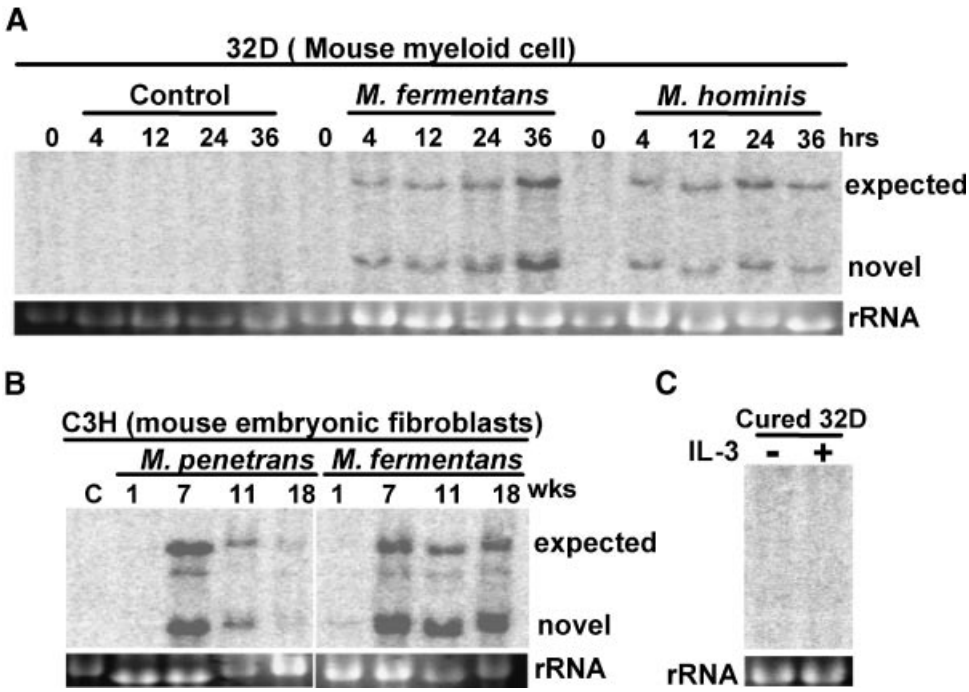

Fig. 2. Mycoplasma infection induces *BMP2* mRNA in mouse cells. Northern blot of *BMP2* mRNA from mycoplasma-positive or -negative cells. **A**: 32D cells were infected with *M. fermentans* or *M. hominis* for 0, 4, 12, 24, and 36 h. **B**: $C_3H/10T_{1/2}$ (**C_3H**) cells were infected with *M. penetrans* or *M. fermentans* for 1, 7, 11, and 18 weeks. "**C**" is control uninfected cells. **C**: 32D cells were transformed by *M. fermentans* then cured by ciprofloxacin. Cells were grown in the presence or absence of IL-3. Ethidium bromide stained rRNA was used as a loading control.

mycoplasma was eradicated from the mycoplasma-transformed cells (Fig. 2C). This suggests that the presence of mycoplasma in the cell culture, but not transformation, explains the *BMP2* induction.

Mycoplasma Induces *BMP2* Expression Via Post-Transcriptional Mechanisms

Mycoplasma induces *BMP2* gene expression by unknown mechanisms. We have identified murine and primate sequences flanking the *BMP2* distal promoter that mediate appropriate gene regulation in response to inducing agents in mouse cells [Abrams et al., 2004]. Although *BMP2* RNA and protein is undetectable in uninfected BEAS-2B cells, the human minimal promoter is clearly active (Fig. 3A). Furthermore, despite the dramatic upregulation of *BMP2* following mycoplasma infection (Figs. 1 and 2), reporter genes containing the *BMP2* promoter region were expressed equally in uninfected or infected BEAS-2B cells (Fig. 3A). This supports the hypothesis that post-transcriptional mechanisms mediate *BMP2* induction in BEAS-2B cells in response to mycoplasma.

We have demonstrated that an ultra-conserved sequence in the 3′ untranslated region (UTR) of *BMP2* is a potent post-transcriptional regulator [Abrams et al., 2004; Fritz et al., 2004b, 2006; Hu et al., 2006]. We performed UV cross-linking experiments to begin to characterize the *BMP2* RNA-binding factors that may influence *BMP2* expression. Distinct RNA:protein interactions occur in mycoplasma-infected BEAS-2B or A549 cells relative to uninfected cells (Fig. 3B). 3′UTR-binding proteins can influence translation, or cytoplasmic degradation or both. We have previously reported that *BMP2* is regulated at the level of RNA stability in other cells [Fritz et al., 2004b]. Therefore, we tested the in vitro half-life of *BMP2* RNA in cell extracts from mycoplasma-infected or uninfected BEAS-2B cells. *BMP2* RNAs are more stable in extracts from mycoplasma-infected cells relative to extracts from uninfected cells (Fig. 3C). An increased half-life would at least partly explain the elevated *BMP2* RNA level in infected cells.

We have begun to characterize the proteins that bind the ultra-conserved 3′UTR element

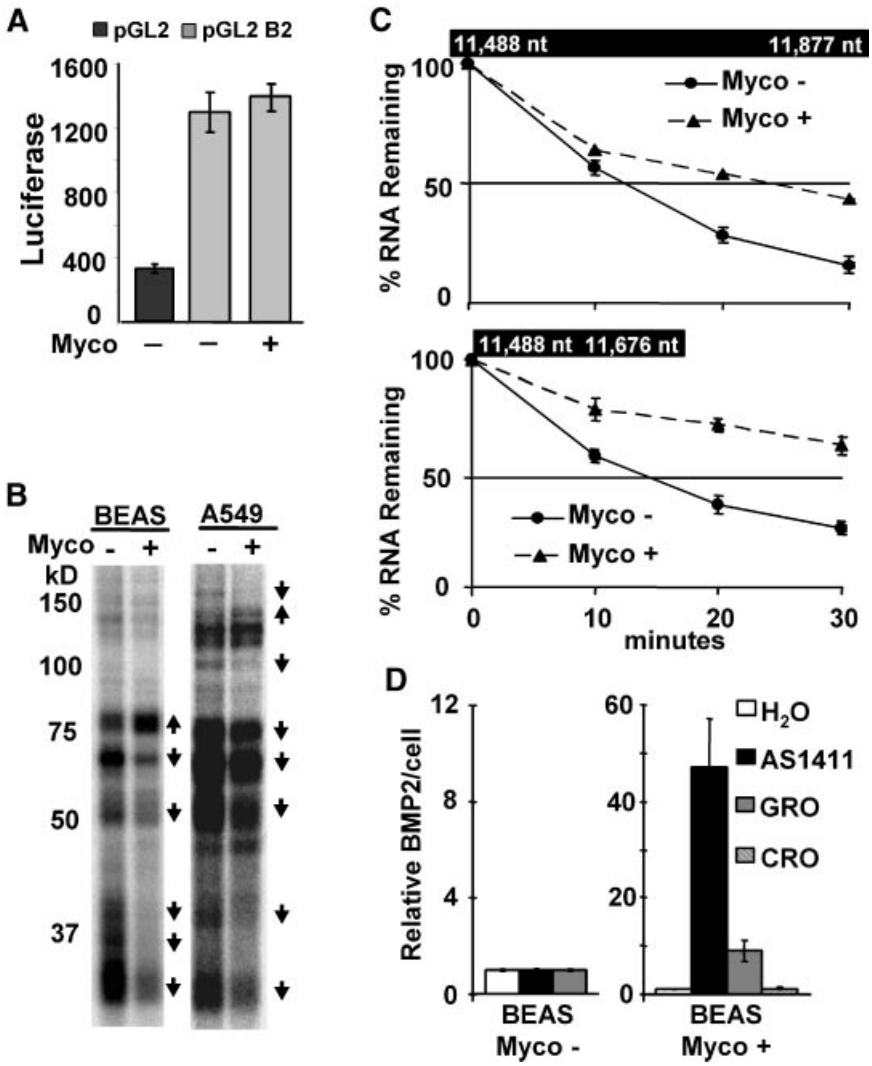

Fig. 3. Mycoplasma infection affects *BMP2* expression by post-transcriptional mechanisms. **A**: Mycoplasma-infection does not affect *BMP2* promoter activity. A reporter construct with the human *BMP2* distal promoter (from nt −147 to 494 relative to distal promoter [Abrams et al., 2004]) upstream of the luciferase (**pGL2 B2**) was transfected into uninfected or mycoplasma (**Myco**)-infected BEAS-2B cells. The empty vector (**pGL2)** was transfected into uninfected cells. After 24 h of transfection, cells were lysed and luciferase activity was measured. Luciferase values were normalized to total protein amount in the transfected cells. Average reporter activity is shown ± SEM, n = 3–4. **B**: Mycoplasma-infection changes the profiles of proteins that bind the *BMP2* RNA. ^{32}P-labeled *BMP2* RNAs with an ultra-conserved human *BMP2* 3′UTR sequence (nt. 11,488–nt. 11,877 relative to the distal promoter, [Abrams et al., 2004; Fritz et al., 2004b]) were UV cross-linked in cytosolic extracts from uninfected or mycoplasma-infected BEAS-2B cells (**BEAS**) or **A549** cells with excess polyA competitor. The mycoplasma infection changed the relative intensity of many proteins labeled by UV cross-linking to the *BMP2* RNA. Arrows indicate decreased or increased labeling relative to the uninfected cell extracts. **C**: *BMP2* RNAs were more stable in extracts from the infected cells which express *BMP2*. Capped and ^{32}P-labeled RNAs with the full-length ultra-conserved *BMP2* sequence (nt. 11,488–nt. 11,877) or approximately the first-half of the conserved *BMP2* sequence (nt. 11,488–nt. 11,676) were incubated in extracts from uninfected (**Myco**−) or mycoplasma (**Myco**+)-infected BEAS-2B cells with excess polyA competitor. Intact RNA was plotted as a % of RNA in unincubated extracts at time 0. n = 1–4. **D**: A nucleolin-binding drug (AS1411) induces *BMP2* in mycoplasma-infected cells, but not uninfected cells. Three days treatment with 10 μM AS1411 (aka AGRO100), an ''antiproliferative'', G-rich oligonucleotide (GGTGGTGGTGGTTGTGGTGGTGGTGG) induced the BMP2 protein in mycoplasma-transformed BEAS-2B cells, but not in normal BEAS-2B cells. As controls, cells were treated with GRO, an inactive G-rich oligonucleotide (TTGGGGGGGGTGGGT); CRO, an inactive C-rich oligonucleotide (CCTCCTCCTCCTTCTCCTCCTCCTCC) or water. Average BMP2 protein level is shown ± SEM, n = 2–5.

[Fritz et al., 2006]. One of these proteins is nucleolin ([Fritz et al., 2006], Jiang and Rogers, in preparation). This multifunctional, shuttling protein, first shown to be necessary for ribosome biosynthesis, has since been localized to many other cell compartments and to be induced in malignant cells [Srivastava and Pollard, 1999; Mongelard and Bouvet, 2007]. In the cytoplasm, nucleolin influences the stability of many mRNAs [Chen et al., 2000; Singh et al., 2004; Otake et al., 2005; Jiang et al., 2006; Zhang et al., 2006b]. A new class of chemotherapeutic drugs now in Phase II clinical trials against NSCLC and other cancers (anti-proliferative G-rich oligonucleotides (GROs, [Laber et al., 2005; Ireson and Kelland, 2006], *http://www.antisoma.com/asm/*), are thought to act at least partly through nucleolin [Bates et al., 1999]. We compared BMP2 protein levels in infected and uninfected cells treated with the anti-proliferative GRO, AS1411, or two control oligonucleotides (GRO and CRO) that neither bind nucleolin nor repress cell growth. None of these compounds induced BMP2 in uninfected cells. In contrast, BMP2 levels were strikingly elevated in the infected cells treated with AS 1411 but not the control compounds (Fig. 3D). This suggests that mycoplasma infection alters the effect of RNA binding proteins, such as nucleolin, that control BMP2 expression.

Mycoplasma Infection Caused Morphological Changes in BEAS-2B Cells

Mycoplasma infection can immortalize host cells and even induce transformation in mammalian cells [Tsai et al., 1995; Feng et al., 1999; Zhang et al., 2004]. The immortalized human bronchial cell line BEAS-2B grows in a contact-inhibited manner. BEAS-2B cells differentiate and die when they are confluent. After 5 days of exposure with conditioned-medium from mycoplasma-positive cells, some BEAS-2B cells started to form cellular outgrowths and began to aggregate (data not shown). After 10 days post-infection, some cells changed morphology from flat epithelial cells (Fig. 4A) to round and small (Fig. 4B). After 44 days, more than half of the cells changed morphology and piled up in clumps resembling foci (Fig. 4C). Although BEAS-2B cells have been shown to transform spontaneously after 32 passages [Reddel et al., 1993], these changes were never observed in uninfected cells (Fig. 4A) grown in parallel. One of the major characteristics of transformation is the loss of contact inhibition. The loss of contact inhibition suggests mycoplasma infection may transform BEAS-2B cells.

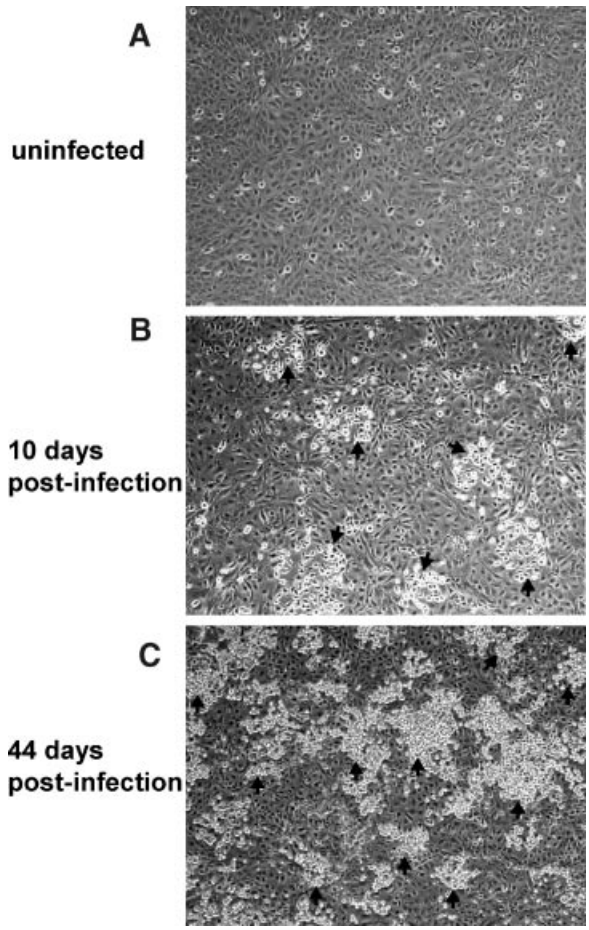

Fig. 4. Mycoplasma infection changes BEAS-2B cell morphology. Control uninfected BEAS-2B cells (**A**), or BEAS-2B cells that had been infected with mycoplasma and cultured for 10 days (**B**), or 44 days (**C**), were plated at the same density (530 cells/cm^2) and grown in parallel for 5 days. The cells were photographed using a phase contrast microscope (100×). Arrows indicate where the cells formed foci. Foci are never observed in uninfected cultures.

Chronic Mycoplasma Infection in BEAS-2B Cells Induced Anchorage-Independent Growth in Soft Agar

Anchorage-independent growth in soft agar is a hallmark of oncogenic transformation. To confirm that the mycoplasma-infected cells were transformed, we examined their ability to form colonies in soft agar. The same numbers (1,000, 2,000, 4,000, and 8,000 cells) of uninfected and mycoplasma-infected BEAS-2B cells were cultured in soft agar. After 10 days, more than 70% of the infected BEAS-2B cells seeded

in the plate formed large colonies (Fig. 5A,B). The infected BEAS-2B cells formed colonies at all cell numbers seeded in the plates. In contrast, the uninfected BEAS-2B cells did not form any colonies at any cell number seeded (Fig. 5A,B). Loss of contact inhibition and the ability of the infected BEAS-2B cells to grow aggressively when suspended in soft agar indicate that chronic mycoplasma-infection transformed the BEAS-2B cells.

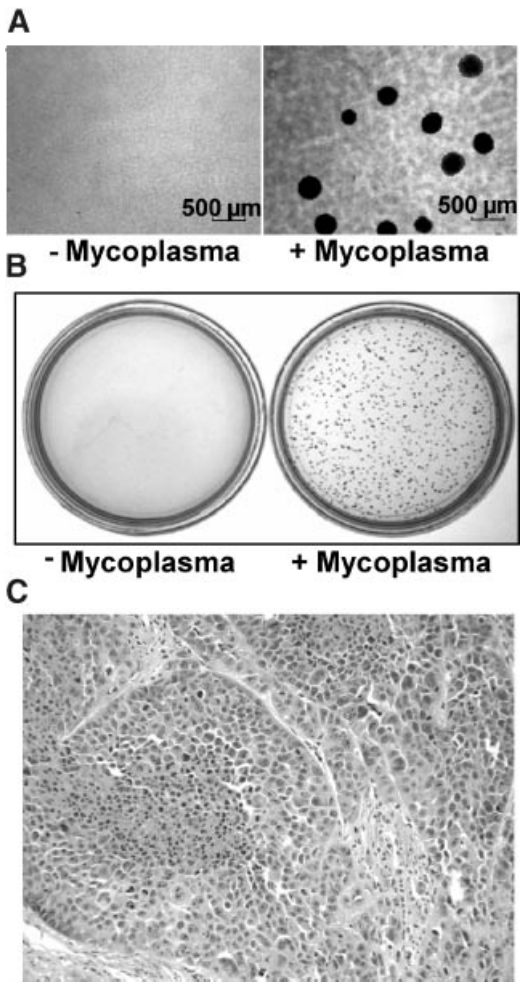

Fig. 5. Mycoplasma infection transformed BEAS-2B cell. Control uninfected BEAS-2B cells, or BEAS-2B cells that had been infected with mycoplasma for 12 weeks were plated at the same number in soft agar and grown in parallel for 10 days. **A**: Representative pictures of cells photographed using a phase contrast microscope (40×). **B**: Representative pictures of plates in which cells were stained with neutral red and photographed using a Gene Bio Imaging system (1×). **C**: Hematoxylin and eosin-stained section of a tumor formed after injection of BEAS-2B cells that had been infected with mycoplasma for 12 weeks (20×). Tumor was removed 8 weeks after injection. No tumors had formed in animals injected with control, uninfected cells by 14 weeks.

Chronic Mycoplasma Infection Caused BEAS-2B Cells to Form Tumors in Nude Mice

BEAS-2B cells are immortalized, but not transformed, and do not form tumors readily in vivo. To assess the effects of mycoplasma infection on tumor growth in vivo, 10^7 uninfected BEAS-2B cells or cells transformed by exposure to mycoplasma for 12 weeks (Fig. 5C) were injected subcutaneously into each of three female NCJ athymic nude mice. Two weeks after injection, palpable tumors were observed exclusively in the three mice injected with mycoplasma-infected cells. Histological analysis of tumors, removed 8 weeks after injection, indicated that the tumor appeared poorly differentiated, highly malignant, and invasive. Mitotic cells and cells with pleiomorphic nuclei were observed frequently as well as regions of necrosis (Fig. 5C). The volumes of these tumors averaged $818 \pm 215\ mm^3$. Animals injected with the uninfected BEAS-2B cells had not formed tumors by the date of euthanasia, 14 weeks after injection. These results show that mycoplasma infection stimulated the progression of these immortalized cells through both transformation and tumorigenicity.

Mycoplasma Infection Increased BEAS-2B Proliferation

Increased growth rate is another characteristic of transformation. We examined if mycoplasma infection increased cell proliferation in BEAS-2B cells. The same number of uninfected cells and infected cells were plated at day 0. Cell number was counted at day 3, 4, 5, 6, and 7. Infected cells grew significantly faster than the uninfected cells (Fig. 6A), resulting in over three times as many cells by the end of a week (Fig. 6A).

Noggin Inhibited the Rapid Growth of the Mycoplasma-Infected BEAS-2B Cells

Mycoplasma induced BMP2 protein in BEAS-2B cells (Fig. 1). BMP2 induces cell proliferation in lung cancer cells in vivo [Langenfeld et al., 2003, 2006]. We hypothesized that increased BMP2 induced cell proliferation in infected BEAS-2B cells. We compared the relative growth of untreated cells or cells treated with recombinant human Noggin, a well-characterized BMP antagonist [Canalis et al., 2003]. Mycoplasma-infected BEAS-2B or uninfected cells were

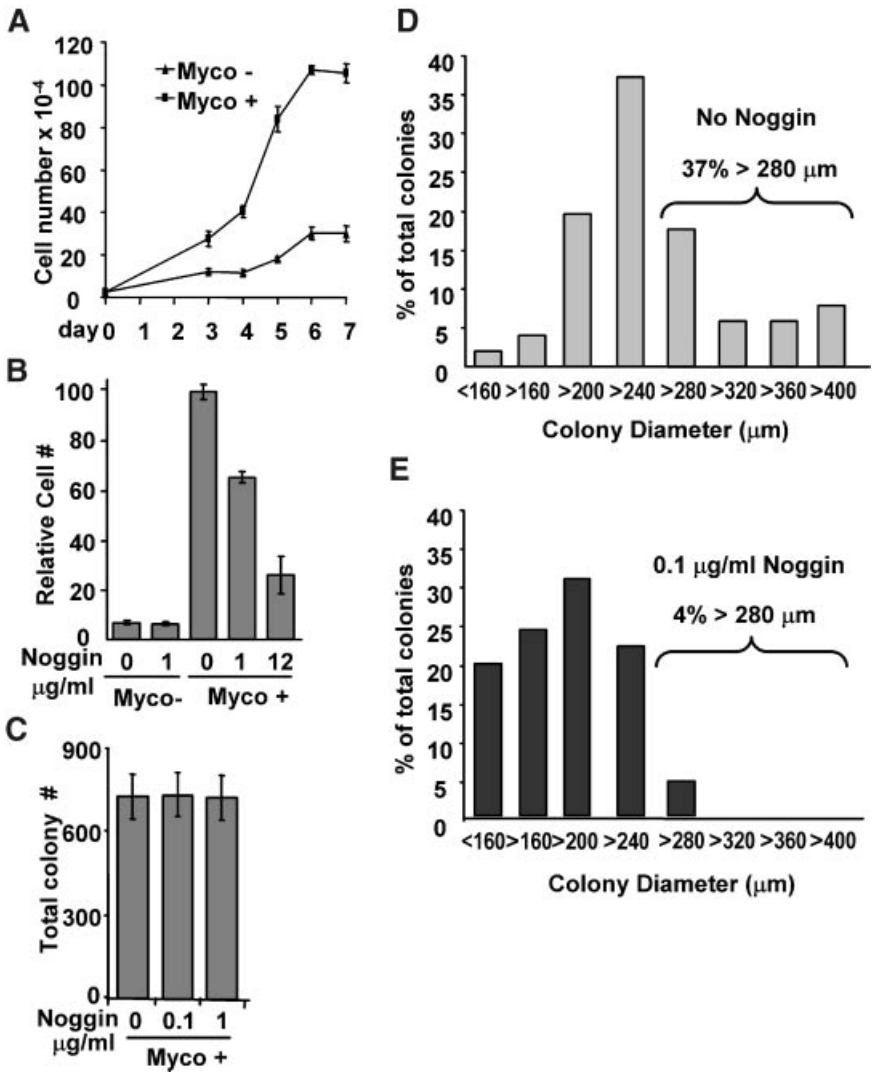

Fig. 6. Mycoplasma infection increased the proliferation of BEAS-2B cells and Noggin inhibited the induced proliferation. **A**: The same number of uninfected (**Myco**−) or mycoplasma infected (**Myco**+) BEAS-2B cells were plated in monolayer at day 0. Cells were counted on day 3, 4, 5, 6, and 7 to determine the growth rate (n = 3). **B**: The same number of uninfected (**Myco**−) or mycoplasma infected (**Myco**+) BEAS-2B cells were grown in monolayer in the presence of 0, 1, or 12 mg/ml recombinant human Noggin as indicated. The number of cells at day 4 was graphed relative to the infected cells without drug treatment (n = 2–6). **C**: Mycoplasma-infected BEAS-2B cells (1×10^3) were plated in a soft agar in the presence of 0, 0.1, or 1 μg/ml human recombinant Noggin and cultured for 10 days (n = 3). Noggin treatment did not change the total number of colonies larger than 100 μm. **D**,**E**: The diameters of untreated (D) or Noggin-treated (E) colonies described above were measured. The average diameters ± SD of the untreated or Noggin-treated colonies were 265 ± 65 μm (n = 51) and 192 ± 51 μm (n = 46), respectively. A Student's *t*-test (two-sample assuming unequal variances) comparing the size of Noggin treated versus untreated colonies indicated that the size difference was significant, $P = 1.8 \times 10^{-8}$. To illustrate the strongly growth repressive action of Noggin in soft agar, the percent of colonies in each treatment category within 40 μm size ranges are shown.

treated with 0, 1, or 12 μg/ml Noggin for 4 days and counted to compare relative growth. Noggin significantly reversed the growth-promoting effect of mycoplasma-infection, but did not affect the uninfected cells (Fig. 6B). This supports the hypothesis that aberrant *BMP2* expression influences BEAS-2B cell proliferation.

We also tested if BMP2 affects the anchorage-independent growth of the infected BEAS-2B cells. Mycoplasma-infected BEAS-2B cells (1×10^3) were seeded in soft agar with or without 0.1 or 1 μg/ml Noggin for 10 days. Colonies larger than 100 μm in diameter were counted. The number of colonies in soft agar with Noggin was the same as without Noggin (Fig. 6C, no Noggin, 728 ± 82 colonies; 0.1 μg/ml Noggin, 733 ± 77 colonies; 1 μg/ml Noggin, 731 ± 82 colonies, n = 3). This suggests that BMP2 does

not mediate the ability to grow without attachment per se and therefore does not reverse transformation. However, consistent with the anti-proliferative effect in monolayer, Noggin significantly reduced the average size of the colonies (compare Fig. 6D–E). The mean diameter of the untreated colonies (263 μM ± 65 μm, n = 51) was significantly greater than that of colonies treated with the 0.1 μg/ml Noggin (192 ± 51 μm, n = 46, $P = 1.8 \times 10^{-8}$). Furthermore, Noggin nearly abolished the formation of any colonies larger than 280 μM (Fig. 6E). The growth inhibitory effect of Noggin on attached or suspended cells indicates that BMP2 promotes the proliferation rate of mycoplasma-transformed human BEAS-2B cells.

DISCUSSION

Cell cultures (15–80%) may be mycoplasma-contaminated [Fleckenstein et al., 1994; Uphoff and Drexler, 2002]. Mycoplasma infection can significantly alter signaling and gene expression patterns in cultured cells [Zhang et al., 2000; Yang et al., 2002; Miller et al., 2003]. Here, we report that mycoplasma infection significantly altered the expression of *BMP2*, a growth factor that regulates many essential biological activities. Also, we report that mycoplasma infection induced the expected full length and a novel shorter *BMP2* mRNA in diverse cell types (mesenchymal, epithelial, and myeloid). The novel transcript may not be detected by other commonly used methods, for example, reverse transcription-PCR (RT-PCR) and RNase protection assays, which detect only part of the transcript. This observation suggests that the *BMP2* gene may yield a previously unknown gene product in some situations.

Diverse agents have been shown to induce BMP2 in different cell types to greater or lesser extents. BMP2 itself, estrogens, bisphosphonates, and statins induce BMP2 modestly (from twofold to fourfold [Mundy et al., 1999; Ghosh-Choudhury et al., 2001; Zhou et al., 2003; Im et al., 2004]). In contrast, inflammatory cytokines [Fukui et al., 2003; Lories et al., 2003; Fukui et al., 2006] and retinoids [Rogers et al., 1992; Rogers, 1996; Helvering et al., 2000] have been shown to induce BMP2 10–35-fold. By comparison to our own studies using retinoic acid and to published studies using other chemical compounds, mycoplasma stands out as a robust and general experimental inducer of BMP2 expression. Furthermore, our discovery that an ultra-conserved sequence in the 3′UTR plays a role in mycoplasma-induced BMP2 upregulation is consistent with a growing body of data from our lab and others indicating that post-transcriptional processes are essential aspect of BMP2 gene regulation [Abrams et al., 2004; Fritz et al., 2004a, 2006; Fukui et al., 2006; Hu et al., 2006].

BMP2 is a classic morphogen whose potent influence on cells from the embryo to the adult changes with concentration. Depending on cell type, BMP2 can induce as well as repress apoptosis and cell proliferation [Glozak and Rogers, 1996; Chen et al., 2001; Hay et al., 2001; Izumi et al., 2001; Hallahan et al., 2003; Zhang et al., 2003; Sugimori et al., 2005]. The role of BMP2 concentration per se has been extensively studied in cell culture and in embryo or organ cultures (e.g., [Katagiri et al., 1990; Wang et al., 1993; Schlange et al., 2000; Schlueter et al., 2006]). Intense effort also is being devoted to identifying cell- and virus-based systems to deliver BMP2 for orthopedic uses [Reddi, 2001]. Undetected mycoplasma contamination would confound the interpretation of how BMP2 functions in lab models of growth, differentiation, and apoptosis and the application of BMP2 in orthopedic settings.

Mycoplasma infection is likely to influence the onset or progression of pathologies in tissues that respond to BMP2. We are the first to report that chronic mycoplasma infection causes transformation and tumorigenicity in a normal human lung cell line (BEAS-2B, Figs. 4–6). This further confirms our previous observations that certain mycoplasmal species can transform mammalian cells [Tsai et al., 1995; Feng et al., 1999; Zhang et al., 2004]. Furthermore, mycoplasma infection induced mature BMP2 protein expression in both normal and malignant lung cells. Human lung cancer carcinomas have raised BMP2 levels [Langenfeld et al., 2003, 2005]. Additionally, experimentally increased BMP2 levels promote lung tumor formation and metastasis in mice [Feeley et al., 2006; Langenfeld et al., 2006]. Our previous studies suggested that BMP2 promotes lung tumor growth by increasing cell proliferation, migration, and angiogenesis [Langenfeld et al., 2003, 2006; Langenfeld and Langenfeld, 2004]. If mycoplasma also induces *BMP2* expression in vivo, then this may influence the onset and progression of lung carcinomas. Our findings also may

have broader oncology implications in other BMP2-responsive tissues that may be colonized by mycoplasma, such as the urogenital tract [Waites et al., 2005].

Clearly, any pathogen that strongly induces BMP2 expression in both normal and malignant lung cells is clinically relevant. The incidence of mycoplasma infection in NSCLC patients is unknown although a limited study suggested that small cell lung cancer patients were infected more frequently than non-cancer patients [Pehlivan et al., 2004]. We found that 11 out of 11 tumor samples from NSCLC patients contained detectable mycoplasma. These results demonstrate that NSCLC patients without clinically significant mycoplasma infections can have occult mycoplasma in their tumors. Furthermore, antibiotics that kill mycoplasma can improve NSCLC survival [Mikasa et al., 1997]. Our study directly links mycoplasma infection to BMP2 induction and suggests that the anti-mycoplasma treatment may be beneficial for lung cancer patients. Furthermore, the finding that AS1411 induced BMP2 synthesis exclusively in mycoplasma-infected cells strongly suggests that mycoplasma-infection would significantly alter the effect of this chemotherapeutic drug in lung cells, and potentially other target cells.

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