

Up-Regulation of EphB4 in Mesothelioma and Its Biological Significance

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Abstract Purpose: Mesothelioma is a rare malignancy that is incurable and carries a short survival despite surgery, radiation, or chemotherapy. This study was designed to identify novel targets for diagnostic, prognostic, and therapeutic approaches.

Experimental Design: The expression and functional significance of the receptor tyrosine kinase EphB4 was studied *in vitro* and in a murine model of mesothelioma.

Results: EphB4 was highly expressed in mesothelioma cell lines and primary tumor tissues but not in normal mesothelium. Knockdown of EphB4 using small interfering RNA and antisense oligodeoxynucleotide showed reduction in cell survival, migration, and invasion. EphB4 knockdown initiated a caspase-8-mediated apoptosis and down-regulation of the antiapoptotic protein bcl-xl. EphB4 knockdown also resulted in reduced phosphorylation of Akt and down-regulation of matrix metalloproteinase-2 transcription. In addition, murine tumor xenograft studies using EphB4 oligodeoxynucleotides showed a marked reduction in tumor growth accompanied by a specific decline in EphB4 protein levels, reduced cell division, apoptosis in tumor tissue, and decreased microvascular density.

Conclusions: EphB4 is expressed in mesothelioma, provides a survival advantage to tumor cells, and is therefore a potential novel therapeutic target.

Mesothelioma is a rare neoplasm that most often arises from the serous surface of the pleural and peritoneal cavity (1). There are three main histologic types of malignant mesothelioma (i.e., epitheloid, mixed or biphasic, and sarcomatoid), with survival being higher with the epitheloid and shorter with the sarcomatoid type (2). There is a strong association with asbestos exposure with ~80% of malignant mesothelioma occurring in individuals who have ingested or inhaled asbestos. The disease has an incidence of 3,000 cases per year in United States with 2,500 deaths ascribed to it (3). Most patients present with locally advanced tumor that is incurable with surgery or radiation therapy (4–6). Response to immune modulatory therapy, such as interleukin-2 or IFN, has also been limited (7). Hence, most patients with advanced disease are treated with systemic chemotherapy. Agents with activity in mesothelioma include anthracyclines, cisplatin, Gemzar, and, more recently, pemetrexed (8). A large, randomized trial

combining pemetrexed and cisplatin compared favorably with a response in 41.3% versus 16.7% response following cisplatin treatment alone, associated with median duration to progression of 5.7 versus 3.9 months. Although there was an increase in survival to a median of 12 months compared with 9 months for cisplatin alone, long-term survival remained dismal (9). Thus, new treatment approaches are needed with an emphasis on targeted therapeutics.

Progress has been made in identifying alterations in gene expression in mesothelioma to better understand tumor cell biology and identify novel therapeutic targets. For example, we and others have shown that vascular endothelial growth factor (VEGF) and VEGF-C function as autocrine growth factors for mesothelioma (10, 11). VEGF inhibitors, including neutralizing antibodies and antisense molecules, are currently in clinical investigations (12). Epidermal growth factor receptor (EGFR) and platelet-derived growth factor (PDGF) are also expressed in mesothelioma and could provide growth advantage to tumor cells (13–16). The potential benefit of EGFR and PDGF inhibitors is also the focus of clinical trials (17, 18).

To discover additional molecular therapeutic targets that might be incorporated into a multimodality regimen for the treatment of mesothelioma, we studied the expression and function of the receptor tyrosine kinase (RTK) EphB4 and its cognate ligand, EphrinB2. EphB4 is a member of the largest family of RTKs and plays an important role in a variety of processes during embryonic development, including pattern formation, cell aggregation and migration, segmentation, neural development, angiogenesis, and vascular hierarchical remodeling (19–22). EphrinB2, the transmembrane ligand of EphB4, also participates in vascular remodeling via reverse

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signaling. Targeted knockout of either EphB4 or EphrinB2 in mice shows similar phenotypes with disrupted vessel maturation and early embryonic lethality (20). This receptor-ligand pair is not only important in vasculogenesis in the embryo but also essential for defining the boundary between arterial and venous domains (23), which persists in the adult life (24). Venous endothelial cells express EphB4, whereas arterial endothelial cells express EphrinB2 (20, 23). In addition to their role in embryonic development, recent studies have shown that they are implicated in tumor development (25). Aberrant expression of EphB4 in cancer has been suggested in colon cancer, breast cancer, and endometrial carcinoma (26–30).

In the current study, we first show the expression of various receptors of the EphB family and their ligands in mesothelioma. We then show that EphB4 plays an important role in cell survival, migration, and invasion. EphB4 knockdown leads to caspase-8-mediated (extrinsic pathway) apoptosis and a reduction in the antiapoptotic protein bcl-xl. Targeting EphB4 *in vivo* led to a specific reduction in EphB4 protein levels that resulted in a significant inhibition in tumor growth combined with reduced mitotic index, tumor cell apoptosis, and reduced microvascular density. Together, these results provide evidence that EphB4 is a novel therapeutic target for the treatment of mesothelioma.

Materials and Methods

Cell lines and reagents. NCI H28, NCI H2052, NCI H2373, and MSTO 211H mesothelioma cell lines, MCF-7 breast cancer cell line, 5637 bladder cancer cell line, and 293T human embryonic kidney cell line were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD) and penicillin/streptomycin (Invitrogen, Carlsbad, CA). 211H cells were cultured on plates coated with fibronectin (50 µg/mL; Sigma, St. Louis, MO). Primary mesothelioma cells were obtained from tumor samples. Tumor biopsy specimens fixed in formalin were also obtained from 16 patients with mesothelioma. Normal pleura and peritoneum were obtained from patients undergoing surgery for unrelated reasons. EphB4-specific small interfering RNAs (siRNA; Table 1) and phosphorothioate-modified antisense oligodeoxynucleotides (Table 2) were synthesized at University of Southern California/Norris Cancer Center Microchemical Core Laboratory. Effective and specific antisense oligodeoxynucleotide (AS-10) and siRNA (siRNA 472) that knockdown EphB4 expression in 293T cells transiently transfected with full-length EphB4 were selected for subsequent study (data not shown). Polyclonal EphrinB2 (P20), EphB1 (Q20), EphB2 (C20), EphB3 (L19), EphB4 (C-16), and EphB6 (N19) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal EphB4 antibodies (M91 and VK138, Vasgene, Inc., Los Angeles) were used in Western blotting and immunoprecipitation. Monoclonal antibody against Ki-67 was obtained from DAKO (Carpinteria, CA), β-actin from Sigma, bcl-2 and bcl-xl from Calbiochem (San Diego, CA), and Mcl-1

Table 1. Oligodeoxynucleotides

Name	Sequence
Scrambled oligodeoxynucleotide	5'-TACCTGAAGGTCAGGCGCAC-3'
EphB4 AS-10	5'-ATGGAGGCCTCGCTCAGAAA-3'

Table 2. siRNAs

Name	siRNA sequence
GFP	5'-CGCUGACCCUGAAGUUCATUU-3' 3'-UUGCGACUGGACUUAAGUA-5'
EphrinB2	5'-GCAGACAGAUGCACUAUUUU-3' 3'-UUCGUCUGUCUACGUGAUAAU-5'
EphB4 472	5'-GGUGAAUGUCAAGACGCGUU-3' 3'-UUCACUUCAGUUCUGCGAC-5'

from BD PharMingen (San Diego, CA). FITC-labeled lycopersicon lectin was obtained from Sigma.

Reverse transcription-PCR. Total RNA was extracted using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX). First-strand cDNA was synthesized from 5 µg total RNA using SuperScript III (Invitrogen). Primer sequences are as follows: EphB4 forward primer 5'-TGA-GGTCACTGCATTGAACGGG-3' and reverse primer 5'-AACTCGCT-CATCCAGTT-3' (product size, 205 bp); EphrinB2 forward primer 5'-AGACAAGAGCCATGAAGATC-3' and reverse primer 5'-ATGATAATGTCCTGGGCTCTGA-3' (product size, 412 bp); and glyceraldehyde-3-phosphate dehydrogenase forward primer 5'-TGAAGTCCGAGTCAACGGATTGGT-3' and reverse primer 5'-CATGTGGGCCATGAGGTCCACCAC-3' (product size, 983 bp). The primers for EphB1, EphB2, EphB3, EphB6, EphrinB1, and EphrinB3 (31) and matrix metalloproteinase (MMP)-2 and MMP-9 (32) were as described previously. PCR was done using AB PCR System 2700 (Applied Biosystems, Foster City, CA). The PCR conditions were 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute. Quantitative reverse transcription-PCR (RT-PCR) was done on the Stratagene MX3000P system (La Jolla, CA) using SYBR Green I Brilliant Mastermix according to the manufacturer's instructions.

Preparation of digoxigenin-labeled RNA probes. EphrinB2 and EphB4 PCR products were cloned using the pGEM-T Easy System (Promega, Madison, WI) according to the manufacturer's description. Primer sequences were 5'-TCCGTGTGGAAGTACTGCTG-3' (forward) and 5'-TCTGGTTTGGCACAGTTGAG-3' (reverse) for EphrinB2 that yielded a 296-bp product and 5'-CTTTGGAAGAGACCCTGCTG-3' (forward) and 5'-AGACGGTGAAGGTCTCCTTG-3' (reverse) for EphB4 that yielded a 297-bp product. The authenticity and insert orientation were confirmed by DNA sequencing. The pGEM-T Easy plasmids containing the PCR product of the human *EphrinB2* or *EphB4* gene were linearized with *SpeI* or *NcoI*. Antisense or sense digoxigenin-labeled RNA probes were transcribed from T7 or SP6 promoters by runoff transcription using a digoxigenin RNA labeling kit (Roche, Indianapolis, IN). RNA probes were quantitated by spot assay as described in the digoxigenin RNA labeling kit instructions.

In situ hybridization. *In situ* hybridization was reformatted as described previously (33). In brief, cells were cultured in Labtech II four-well chamber slides (Nalge Nunc International, Naperville, IL). Cells were washed in PBS at 37°C and fixed for 30 minutes at 25°C in a solution of 4% (w/v) formaldehyde, 5% (v/v) acetic acid, and 0.9% (w/v) NaCl. Slides were rinsed with PBS and stored in 70% ethanol at 4°C until use. Before *in situ* hybridization, cells were dehydrated, washed in 100% xylene to remove residual lipid, and then rehydrated. Cells were permeabilized by incubating at 37°C with 0.1% (w/v) pepsin in 0.1 N HCl for 20 minutes and postfixed in 1% formaldehyde for 10 minutes. Prehybridization was done for 30 minutes at 37°C in a solution of 4× SSC containing 50% (v/v) deionized formamide. Slides were hybridized overnight at 42°C with 25 ng antisense or sense RNA probes in 40% deionized formamide, 10% dextran sulfate, 1× Denhardt's solution, 4× SSC, 10 mmol/L DTT, 1 mg/mL yeast tRNA, and 1 mg/mL denatured and sheared

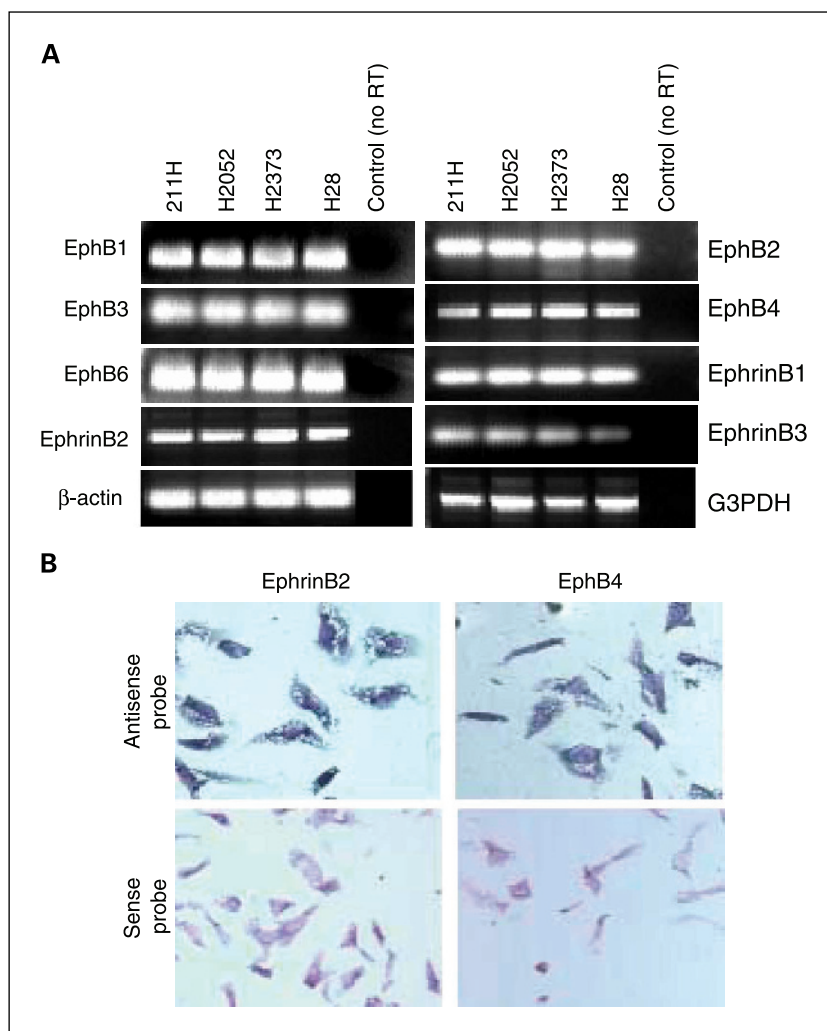
salmon sperm DNA in a total volume of 40 μ L. Slides were then washed at 37°C as follows: twice each for 15 minutes with 2 \times SSC, for 15 minutes with 1 \times SSC, for 15 minutes with 0.5 \times SSC, and for 30 minutes with 0.2 \times SSC. Hybridization signal was detected using alkaline phosphatase-conjugated anti-digoxigenin antibodies (Roche, Indianapolis, IN) according to the manufacturer's instructions. Color development was stopped by two washes in 0.1 mol/L Tris-HCl, 1 mmol/L EDTA (pH 8.0) for 10 minutes. Cells were visualized by counterstaining of nucleic acids with Nuclear Fast Red (Vector Laboratories, Burlingame, CA). Slides were mounted with Immu-Mount (Shandon, Astmoor, United Kingdom).

Western blot. Crude cell lysates were prepared by incubation in cell lysis buffer [10 mmol/L Tris (pH 7.5), 1 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L DTT, 10% glycerol]. Lysates were cleared by centrifugation at 10,000 \times g for 10 minutes. Total protein was determined by Bradford assay (Bio-Rad, Hercules, CA). Samples (20 μ g protein) were fractionated on a 4% to 20% Tris-glycine polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Bio-Rad) by electroblotting. Membranes were blocked with 5% nonfat milk before incubation with primary antibody at 4°C overnight. Secondary antibody conjugated with horseradish peroxidase was applied for 1 hour at room temperature. The membranes were developed using the SuperSignal West Femto Maximum sensitivity chemiluminescent substrate (Pierce, Rockford, IL). To ensure equal loading and transfer of protein, membranes were stripped with Restore Western Blot stripping buffer (Pierce) and re probed with β -actin.

Immunofluorescence studies. Immunofluorescence was done as described previously (33). Cells were cultured on Labtech II four-well chamber slides and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 30 minutes. The slides were rinsed twice in PBS and preincubated with blocking buffer (0.2% Triton X-100, 1% bovine serum albumin in PBS) for 20 minutes. The slides were then incubated with antibodies to EphB4 or EphrinB2 (1:100) in blocking buffer at 4°C overnight. After washing thrice, the slides were incubated with the fluorescein-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI), washed extensively with PBS, and mounted with Vectashield antifade mounting solution (Vector Laboratories). Images were obtained using an Olympus AX70 fluorescence microscope (Melville, NY) and Spot version 2.2.2 (Diagnostic Instruments, Inc., Sterling Heights, MI) digital imaging system.

Immunohistochemistry. Sections (5 μ m) were deparaffinized in xylene and rehydrated in graded ethanol. Antigen retrieval was done in 10 mmol/L sodium citrate (pH 6.0) at 95°C for 10 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The sections were then incubated with the specific primary antibodies (anti-EphB4, 1:50; anti-EphrinB2, 1:100; Ki-67, 1:100) at 4°C overnight. For negative controls, the primary antibodies were replaced with isotype control IgG. Immunostaining was carried out using the Vector Laboratories ABC kit and color was developed with 3,3'-diaminobenzidine (Vector Laboratories). Sections were counterstained with hematoxylin.

Fig. 1. EphB and EphrinB mRNA expression in mesothelioma. **A**, reverse transcription was done on 5 μ g total RNA extracted from cultured cells. RT-PCR shows that mRNA for EphB and EphrinB in all four mesothelioma cell lines, whereas control samples (no reverse transcriptase) show no signal. **B**, *in situ* hybridization for EphB4 and EphrinB2. H28 mesothelioma cells cultured in chamber slides were hybridized with digoxigenin-labeled probes. Positive signal is seen with antisense probes against EphB4 and EphrinB2, whereas sense probes show no signal.



Cell viability assay. Cells were seeded on 48-well plates at a density of $\sim 1 \times 10^4$ cells per well in a total volume of 200 μ L. Following attachment, cells were treated with various concentrations (0-10 μ mol/L) of EphB4 antisense oligodeoxynucleotide (AS-10) or scrambled oligodeoxynucleotide as control. After 3 days, medium was changed and fresh oligodeoxynucleotides were added. Following a further 48-hour incubation, cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (10). EphB4 siRNA or EphrinB2 siRNA (0-100 nmol/L) were introduced into 1×10^4 cells per well in 48-well plates using LipofectAMINE 2000 according to the manufacturer's instructions. Cell viability was assayed by MTT 48 hours following transfection.

Apoptosis ELISA. Apoptosis was studied *in vitro* using the Cell Death Detection ELISA^{plus} kit (Roche, Piscataway, NJ) according to the manufacturer's instructions. Briefly, cells were cultured in 24-well plates to 80% confluence and treated appropriately. After 48 hours, 1×10^4 cells were incubated in 200 μ L lysis buffer. Nuclei were pelleted by centrifugation and the supernatant (20 μ L) containing the mononucleosomes or oligonucleosomes was incubated with anti-histone-biotin and anti-DNA-POD in streptavidin-coated 96-well plate for 2 hours at room temperature. Color was developed with ABST and absorbance at 405 nm was read in a microplate reader (Molecular Devices, Sunnyvale, CA). Apoptosis was detected in deparaffinized sections of animal tumors by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using the *In situ* Cell

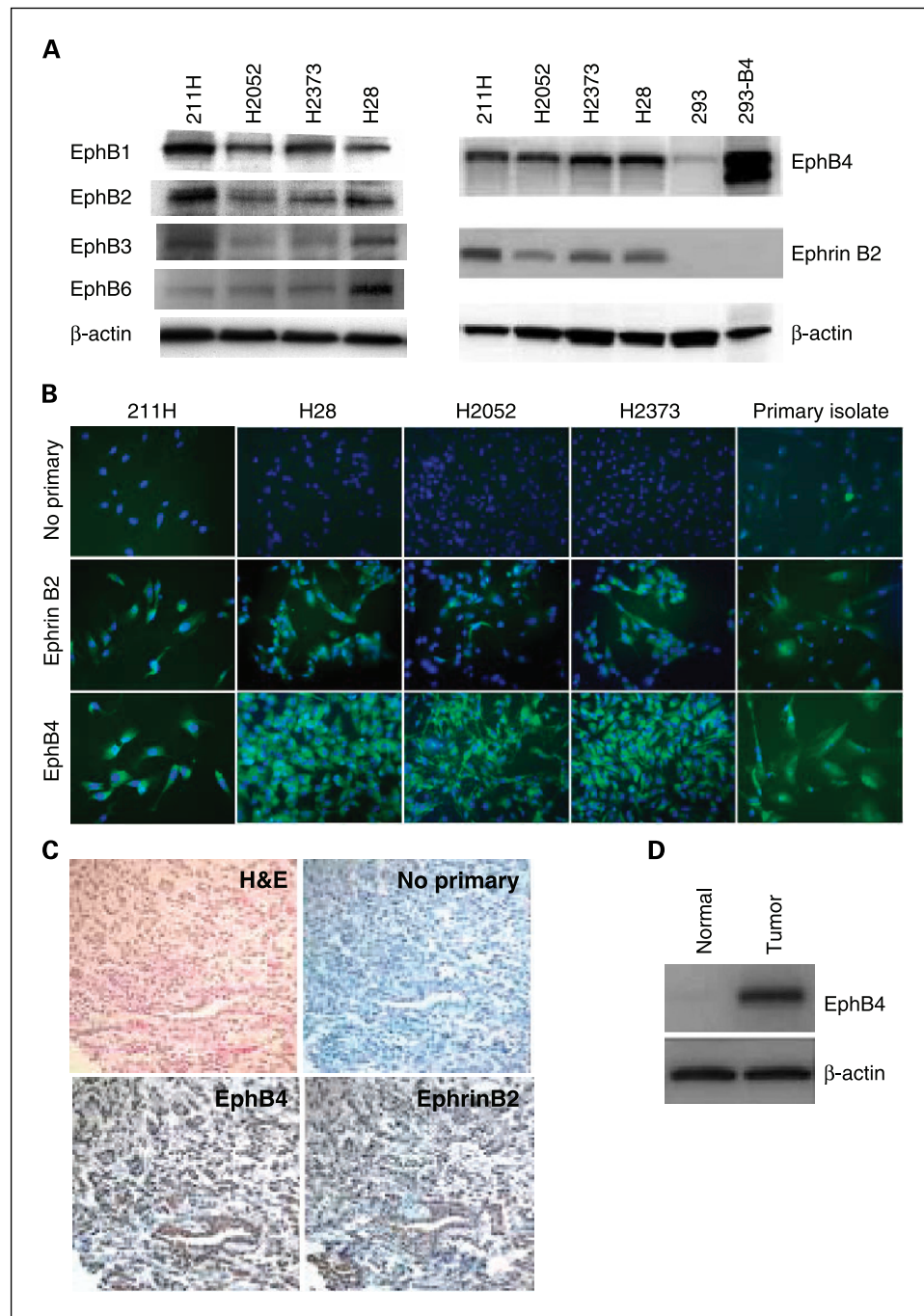


Fig. 2. EphB4 and EphrinB2 protein expression in mesothelioma. *A*, total cell lysate (20 μ g) from each of the mesothelioma cell lines was probed sequentially for EphB1, EphB2, EphB3, EphB4, EphB6, and EphrinB2 expression. 293T cells transiently transfected with empty vector or full-length EphB4 were used as negative and positive controls for EphB4 expression, respectively (*right*). *B*, signals for EphB4 and EphrinB2 were detected by immunofluorescence in mesothelioma cell lines and primary cell isolate from pleural effusion of a patient with malignant mesothelioma. Specificity of immunofluorescence staining is shown by lack of signal with no primary antibody. Original magnification, $\times 200$. *C*, immunohistochemical staining of EphB4 and EphrinB2 on mesothelioma biopsy specimen. H&E staining shows the tumor architecture of mesothelioma. Original magnification, $\times 200$. *D*, protein (20 μ g) extracted from fresh normal peritoneum and mesothelioma tissue was used in a Western blot to detect expression of EphB4.

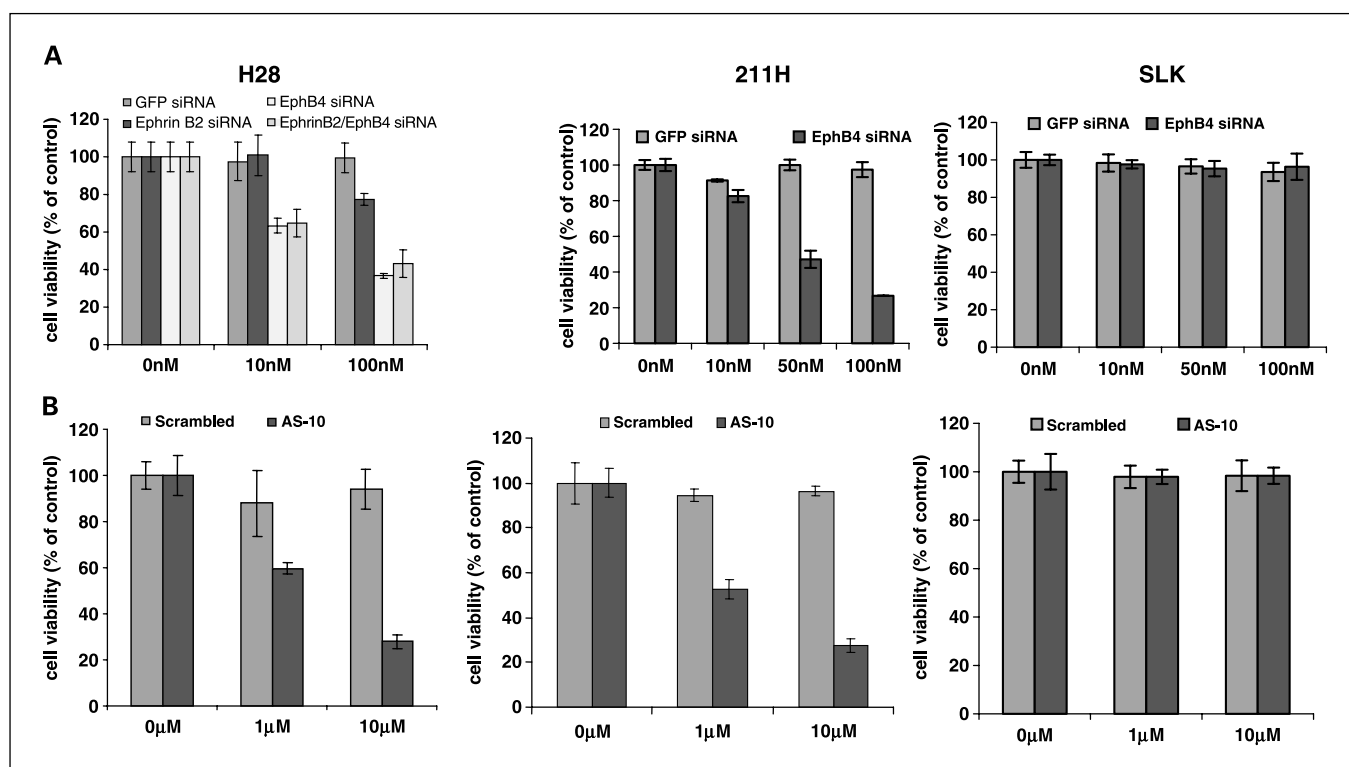


Fig. 3. EphB4 regulates cell viability. *A*, H28 and 211H cells were seeded in equal density on 48-well plates and transfected with different concentrations of EphrinB2 or EphB4 siRNA with LipofectAMINE 2000. Cell viability was evaluated with MTT 48 hours later and expressed as percentage of LipofectAMINE 2000–only treated cells. GFP siRNA was used as control. SLK (Kaposi sarcoma) cells that lack EphB4 expression were used to study the specificity of effect of EphB4 knockdown. *B*, effect of EphB4-specific antisense oligodeoxynucleotide (AS-10) and control scrambled oligodeoxynucleotide on the viability of H28, 211H, and SLK cells was studied following 5 days of treatment.

Death Detection kit (Roche, Piscataway, NJ) according to manufacturer's instructions.

Caspase-8 and caspase-9 activity analysis. Caspase activity was measured by colorimetric assay (R&D Systems, Inc., Minneapolis, MN) according to manufacturer's instructions. Briefly, cells were transfected with EphB4 siRNA or green fluorescent protein (GFP) siRNA, and after 18 hours, cell lysates were incubated with reaction buffer and the appropriate colorimetric substrate at 37°C for 2 hours. Color development was quantified by measuring absorbance at 405 nm. Routine controls included deletion of cell lysate or substrate.

Wound healing migration assay. Cells were seeded onto six-well plates and cultured until confluent. AS-10 or scrambled oligodeoxynucleotide were added to the medium 24 hours before wounding the monolayer by scraping with a sterile pipette tip. To test the effect of siRNA, confluent cultures were transfected with 50 nmol/L EphB4 siRNA or GFP siRNA 12 hours prior wounding. The repopulation of cell-free zone was examined dynamically and recorded with a Nikon Coolpix 5000 digital camera (Melville, NY) with microscope adapter.

Invasion assay. Cells were transfected with EphB4 siRNA or GFP siRNA using LipofectAMINE 2000, and 6 hours later, 0.5×10^5 cells were transferred into 8 µm Matrigel-precoated inserts (BD Bioscience, Palo Alto, CA). The inserts were placed in companion wells containing RPMI 1640 supplemented with 5% FBS and 5 µg/mL fibronectin as a chemoattractant. Following 24-hour incubation, the inserts were removed and the noninvading cells on the upper surface were removed with a cotton swab. The cells on the lower surface of the membrane were fixed in 100% methanol for 15 minutes, air dried, and stained with Giemsa stain for 2 minutes. The cells were counted in five individual high-power fields for each membrane

under a light microscope. Assays were done in triplicate for each treatment group.

Phosphorylation analysis. Cells grown in six-well plates were serum starved (0.1% FBS supplemented RPMI 1640 for 24 hours) and treated with 3 µg/mL EphrinB2/Fc (R&D Systems) or Fc fragment alone (The Jackson Laboratory, Bar Harbor, ME) clustered with anti-human Fc (The Jackson Laboratory) for 60 minutes. Cleared cell lysates were incubated with 2 µg/mL EphB4 monoclonal antibody (VK138) overnight at 4°C. Antigen-antibody complexes were immunoprecipitated by the addition of 20 µL protein G-Sepharose (Santa Cruz Biotechnology) in PBS for 1 hour at room temperature. Immunoprecipitates were analyzed by Western blot with phosphotyrosine-specific antibody (clone 4G10, Upstate, Lake Placid, NY) at 1:1,000 dilution. To monitor immunoprecipitation efficiency, a duplicate membrane was probed with EphB4-specific monoclonal antibody (M91).

In vivo tumor growth studies. Male BALB/c *nu/nu* mice (9 weeks old) were implanted with 5×10^6 cells in the flank. After confirming equal tumor sizes, mice bearing the xenografts were randomly divided into three groups (12 mice per group) and treatment started 5 days after tumor implantation. EphB4 AS-10 or scrambled oligodeoxynucleotide dissolved in sterile physiologic saline (0.9% NaCl) was given i.p. at a dose of 20 mg/kg daily for 3 weeks. The mice were monitored clinically and body weight was measured. Tumor volume was calculated as $0.52 \times \text{length} \times \text{width}^2$, where length and width are the largest and smallest extents of the palpable tumor. FITC-labeled lectin (4.5 mg/kg) was infused into animals 30 minutes before sacrifice. Frozen tumor sections were counterstained with DAPI and microvascular density was evaluated using a fluorescent microscope.

Statistical analysis. Mean tumor volumes and number of cells staining positive on immunohistochemistry were compared using Student's *t* test, with $P < 0.05$ considered significant.

Results

EphB4 and EphrinB2 are expressed in mesothelioma cell lines.

The expression of different EphB receptors and Ephrin ligands in malignant mesothelioma cell lines was determined at the RNA and protein levels. RT-PCR showed that all of the four mesothelioma cell lines express EphB1, EphB2, EphB3, EphB4, EphB6, EphrinB1, EphrinB2, and EphrinB3 (Fig. 1A). We confirmed the expression of EphB4 and EphrinB2 by *in situ* hybridization. Representative data are shown for H28 cell line. Specific signal for EphB4 and EphrinB2 transcripts was detected using antisense probes. Sense probes for both served as negative controls (Fig. 1B).

Protein expression was determined by Western blot in all four cell lines. Varying levels of EphB receptors were expressed in the cells (Fig. 2A, left). We further characterized the expression of EphB4 and EphrinB2. A specific band for EphB4 was seen at 120 kDa and EphrinB2 at 37 kDa on Western blot (Fig. 2A, right) in all four mesothelioma cell lines but not in the negative control 293T human embryonic kidney cell line.

Expression of EphB4 and EphrinB2 was confirmed in all the cell lines cultured on chamber slides by immunofluorescence (Fig. 2B).

Evidence of expression of EphB4 and EphrinB2 in clinical samples. Tumor cells isolated from the pleural effusion of a patient with pleural malignant mesothelioma were spun onto slides and stained for EphB4 and EphrinB2. Both EphB4 and EphrinB2 were observed in freshly isolated cells (Fig. 2B, rightmost lane). To determine whether these results were a true reflection of expression in the diseased state, tumor biopsy samples were subjected to immunohistochemical staining for EphB4 and EphrinB2. Twelve of the 16 cases showed positive staining confined to tumor cells (Fig. 2C). We also analyzed protein expression by Western blot analysis on freshly harvested mesothelioma samples. Whereas tumor specimens showed high levels of EphB4 expression, normal peritoneal tissue showed no expression (Fig. 2D).

EphB4 is involved in the cell growth, apoptosis, migration, and invasion of mesothelioma. To define the significance of expression of EphB4 and EphrinB2 on the biological behavior

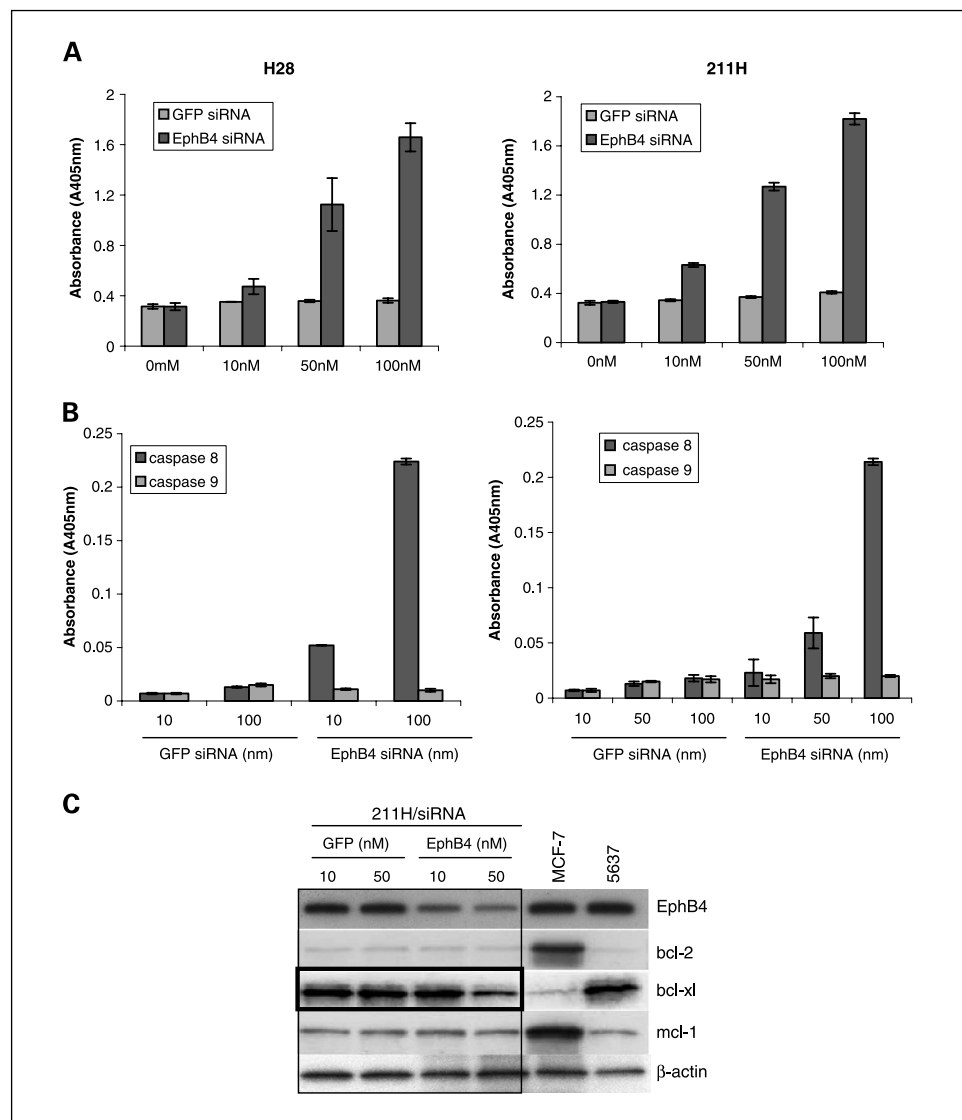
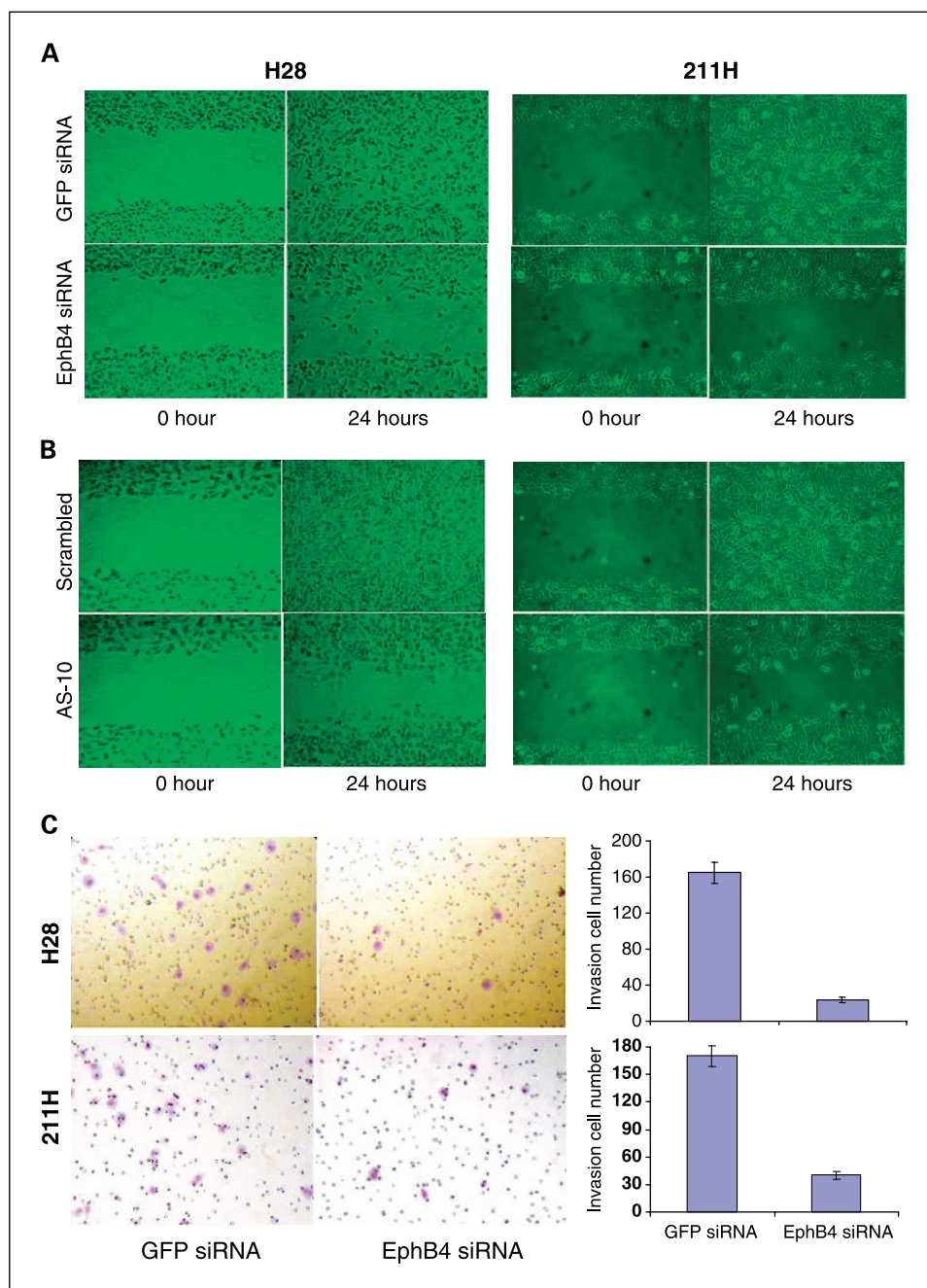


Fig. 4. EphB4 regulates apoptosis in mesothelioma. *A*, H28 cells (left) and 211H cells (right) were transfected with varying concentrations of EphB4-specific siRNA and GFP siRNA. Apoptosis was detected by quantitation of cytoplasmic nucleosomes using the Cell Death Detection ELISA^{plus} kit as described in Materials and Methods. Columns, mean of triplicate samples at the indicated concentrations of EphB4 siRNA and GFP siRNA; bars, SE. *B*, activation of caspase-8 and caspase-9 was assayed colorimetrically in H28 cells (left) and 211H cells (right) transfected with varying concentrations of EphB4 or GFP siRNA. *C*, lysates from 211H cells transfected with varying doses of EphB4 or GFP siRNA were sequentially probed on Western blot for the expression levels of different antiapoptotic factors. MCF-7 (breast cancer) and 5637 (bladder cancer) cells were included as positive control for bcl-2 and bcl-xl, respectively.

Fig. 5. EphB4 regulates cell migration and invasion. **A**, H28 cells (*left*) and 211H cells (*right*) were seeded on six-well plates and cultured until 90% confluent. EphB4 siRNA or GFP siRNA (50 nmol/L each) was transfected into cells with LipofectAMINE 2000. The cell layer was scraped and photographed immediately and after 24 hours. Photomicrographs of representative $\times 20$ fields. **B**, a similar assay was done on H28 cells (*left*) and 211H cells (*right*) treated with AS-10 or sense oligodeoxynucleotide (10 μ mol/L each). Representative photomicrographs were taken immediately and 24 hours after wounding. **C**, Matrigel invasion assay. Invasion of H28 cells (*top*) or 211H cells (*bottom*) into Matrigel was studied in a modified Boyden chamber assay. Cells were transfected with EphB4 siRNA or GFP siRNA (50 nmol/L each). Cells migrating to the underside of the Matrigel-coated insert in response to 5 μ g/mL fibronectin in the lower chamber were fixed and stained with Giemsa. Representative photomicrographs. Cell numbers were counted in five individual high-powered fields. Columns, mean; bars, SE.



of mesothelioma, we first examined their effect on cell growth by knocking down the expression with siRNA. siRNA specific to EphB4 and EphrinB2 were screened in 293T cell line lacking endogenous expression but transiently transfected with expression vectors for EphB4 and EphrinB2. siRNAs that knockdown expression >80% after 48-hour treatment were selected for future use (data not shown). Activity of these active EphB4 and EphrinB2 siRNA was then confirmed in mesothelioma cell lines (data not shown). Transfection of H28 cell line with EphB4 siRNA 472 produced a dose-dependent inhibition of cell viability. Transfection of EphrinB2 siRNA alone did not have the inhibitory effect. Combination of EphB4 and EphrinB2 siRNA did not augment growth inhibition over EphB4 siRNA alone (Fig. 3A, *left*). The EphB4 siRNA was active in a similar

dose-dependent manner in the 211H cell line as well (Fig. 3A, *middle*) but had no effect on the EphB4-negative Kaposi sarcoma cell line, SLK (Fig. 3A, *right*).

The involvement of EphB4 in cell survival was further confirmed with EphB4 antisense oligodeoxynucleotides. Incubation of cultured H28 or 211H cells with EphB4 antisense (AS-10) generated the same dose-dependent inhibition of cell growth (Fig. 3B, *left* and *middle*, respectively). AS-10 had no effect on survival of SLK cells (Fig. 3B, *right*). These results indicate that EphB4 plays a critical role in mesothelioma cell survival.

Apoptosis as a result of EphB4 knockdown was tested by measuring levels of cytoplasmic nucleosomes. A dose-dependent increase in apoptosis was observed when H28 cells (Fig. 4A, *left*) or 211H cells (Fig. 4A, *right*) were transfected with EphB4 siRNA

472, whereas GFP siRNA had no effect. DNA fragmentation in EphB4 siRNA 472-transfected H28 and 211H cells increased nearly 5-fold at a dose of 100 nmol/L when compared with GFP siRNA-treated cells. Induction of apoptosis corresponded to an increase in caspase-8 activity, whereas little change was observed in caspase-9 activity in both H28 cells (Fig. 4B, left) and 211H cells (Fig. 4B, right). 211H cell lysates were also evaluated for levels of antiapoptotic proteins. Knockdown of EphB4 resulted in a significant (60%) decline in bcl-xl levels, whereas bcl-2 and mcl-1 remained unchanged (Fig. 4C). Similar results were obtained with H28 cells (data not shown).

Mesothelioma is a locally advancing disease with frequent extension and growth into adjacent vital structures, such as the chest wall, heart, and esophagus. In an effort to study this process *in vitro*, we did cell migration study with wound healing assay and invasion study with a modified Boyden chamber assay. When a wound was introduced into subconfluent mesothelioma cell culture over the course of 24 hours, cells progressively migrate and repopulate the area of the wound. However, when cells were transfected with EphB4 siRNA (Fig. 5A) or pretreated with AS-10 for 24 hours (Fig. 5B), a marked reduction in cell migration was observed compared with controls. In addition, knockdown of EphB4 expression in H28 or 211H cells with EphB4 siRNA 472 significantly reduced the ability of these cells to invade Matrigel compared with GFP

siRNA treatment (Fig. 5C). Similar results were obtained with AS-10 treatment (data not shown).

EphB4 is biologically active in mesothelioma cell lines. We next examined whether EphB4 is phosphorylated in response to stimulation by ligand (EphrinB2). H28 cells were treated with 3 μ g/mL clustered EphrinB2/Fc for 15 minutes. A low-level basal signal was detected in cells grown under serum-free conditions, and this signal markedly increased following exposure to external clustered ligand (Fig. 6A). Similar results were obtained with 211H cells (data not shown).

To ascertain the downstream effects of EphB4 stimulation, we studied the effect of EphB4 knockdown on the levels of phosphorylated Akt. Treatment with EphB4 siRNA 472 led to an 85% reduction in phosphorylated Akt levels, whereas GFP siRNA treatment had no effect (Fig. 6B). We also evaluated the role of EphB4 on MMP expression by quantitative RT-PCR. EphB4 knockdown by siRNA 472 led to a dose-dependent reduction in mRNA levels of MMP-2 (Fig. 6C) but not MMP-9 (data not shown), whereas GFP siRNA had no effect.

In vivo activity of EphB4 antisense oligodeoxynucleotide in a xenograft model of malignant mesothelioma. H28 and 211H cells were implanted s.c. in the flank of male athymic mice and animals were treated with EphB4 AS-10, scrambled oligodeoxynucleotide, or the diluent (PBS). Tumor volumes were significantly smaller in AS-10 oligodeoxynucleotide-treated group compared with both control groups (Fig. 7A and B; $P < 0.01$). Western blot of extracts of 211H tumor tissue confirmed that the expression of EphB4 in AS-10-treated group was markedly reduced compared with levels in the other treatment groups (Fig. 7B, inset). Furthermore, AS-10 treatment reduced cell proliferative index as measured by Ki-67 staining by 50%, increased apoptosis 7-fold, and reduced microvascular density in the tumor (Fig. 6C). Similar effects were seen in H28 tumors (data not shown).

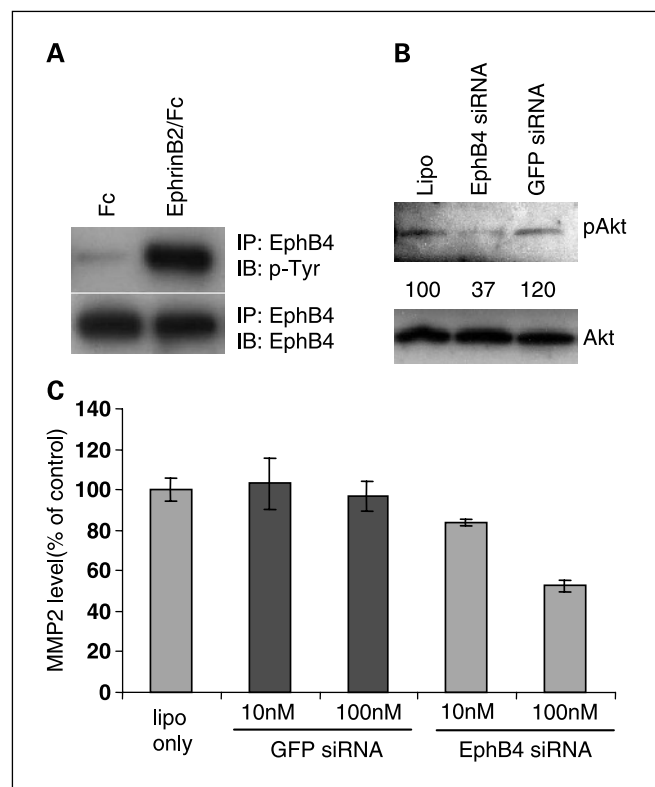


Fig. 6. EphB4 regulates Akt and MMP-2. **A**, H28 cells were serum starved overnight and stimulated with 3 μ g/mL EphrinB2/Fc or Fc alone for 15 minutes. Cell lysates (100 μ g protein) were immunoprecipitated with EphB4 monoclonal antibody and duplicate blots were probed with phosphotyrosine (*p-Tyr*) and EphB4 antibody. **B**, H28 cells were transfected with 50 nmol/L EphB4 or GFP siRNA and cell lysates were probed for phosphorylated and total Akt levels. **C**, H28 cells were transfected with varying doses of EphB4 or GFP siRNA and cellular mRNA was extracted. Level of expression of MMP-2 mRNA was quantitated by real-time RT-PCR.

Discussion

The successful treatment of mesothelioma to improve survival may depend on the identification of novel targets. In the present study, we found that EphB4 is expressed in all three subtypes of mesothelioma (epitheloid, H28 and H2052; sarcomatoid, H2373; and biphasic, 211H). Each cell line showed a different level of EphB4 expression, with the highest level observed in epitheloid type cell line H28. The expression of EphB4 in mesothelioma was confirmed in clinical samples. Due to the small number of cases, we are unable to analyze the relationship between the expression of EphB4 and tumor subtype, grade, and stage. However, studies have shown that overexpression of EphB4 is inversely related to prognosis in endometrial carcinoma, squamous cell carcinoma of the head and neck, and breast cancer (29, 34–36). Further investigation in a large prospective study is required to determine the importance of EphB4 as a marker of progression and metastasis for mesothelioma.

We have shown that EphB4 expressed in mesothelioma cells can be activated by stimulation with EphrinB2, and in tumors, EphB4 stimulation may be similarly achieved by the coexpression of its ligand, EphrinB2. Similar coexpression of this receptor-ligand pair has been shown in other tumor types, including endometrial, lung, and colon carcinoma (30, 31, 37).

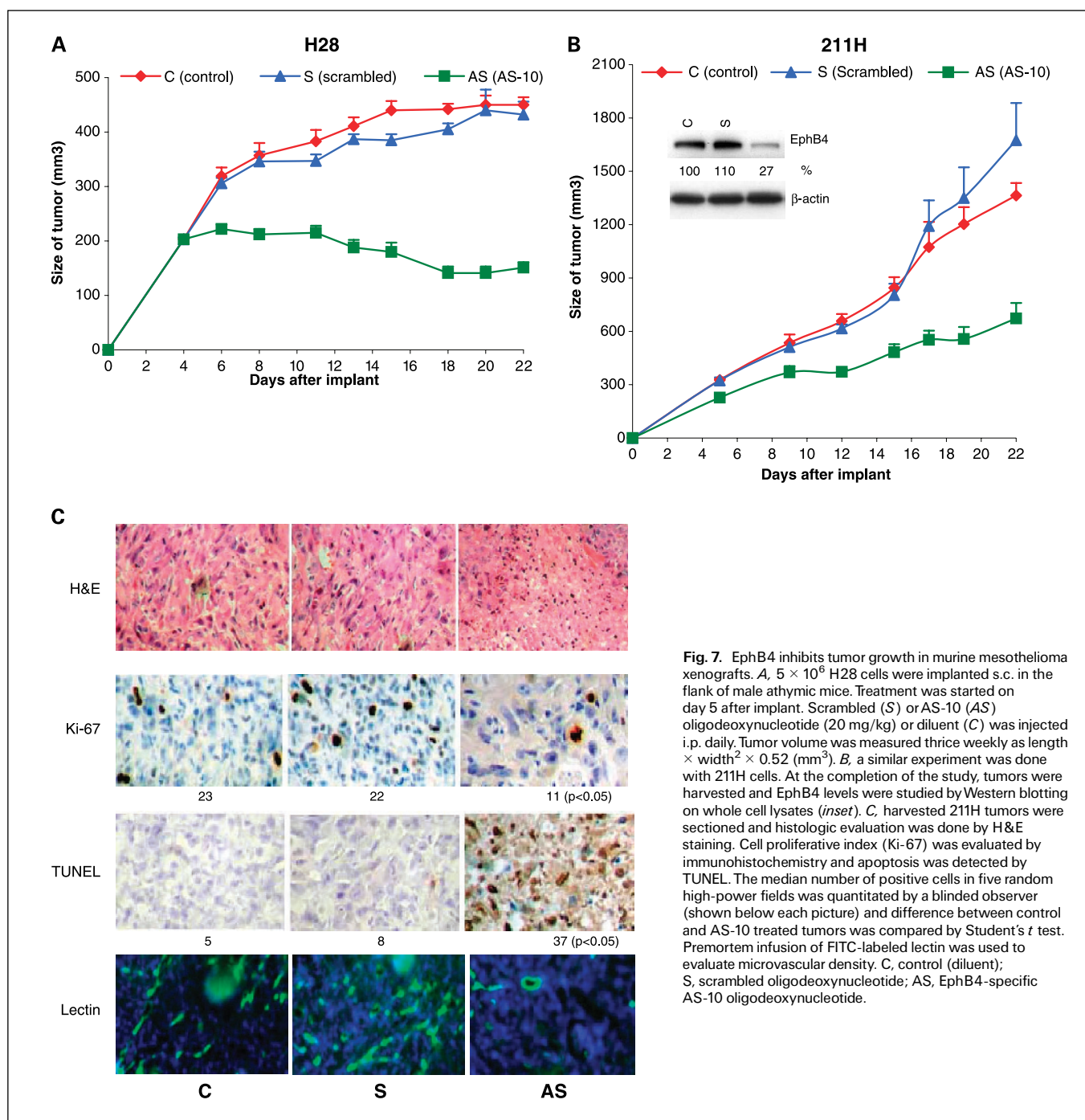


Fig. 7. EphB4 inhibits tumor growth in murine mesothelioma xenografts. *A*, 5×10^6 H28 cells were implanted s.c. in the flank of male athymic mice. Treatment was started on day 5 after implant. Scrambled (S) or AS-10 (AS) oligodeoxynucleotide (20 mg/kg) or diluent (C) was injected i.p. daily. Tumor volume was measured thrice weekly as length \times width² \times 0.52 (mm³). *B*, a similar experiment was done with 211H cells. At the completion of the study, tumors were harvested and EphB4 levels were studied by Western blotting on whole cell lysates (*inset*). *C*, harvested 211H tumors were sectioned and histologic evaluation was done by H&E staining. Cell proliferative index (Ki-67) was evaluated by immunohistochemistry and apoptosis was detected by TUNEL. The median number of positive cells in five random high-power fields was quantitated by a blinded observer (shown below each picture) and difference between control and AS-10 treated tumors was compared by Student's *t* test. Premortem infusion of FITC-labeled lectin was used to evaluate microvascular density. C, control (diluent); S, scrambled oligodeoxynucleotide; AS, EphB4-specific AS-10 oligodeoxynucleotide.

On the other hand, a ligand-independent mechanism is also conceivable, relying on activation as a consequence of overexpression. Overexpression leading to constitutive activation has been observed with EphB2 (38). To determine the possible biological role of EphB4 in mesothelioma, we examined changes in cell viability by targeted disruption of EphB4 expression using antisense and siRNA in two different mesothelioma cells (211H and H28). Down-regulation of EphB4 led to a dose-dependent inhibition in cell viability. The arrest in cell growth was mediated by an increase in apoptosis. The up-regulation of caspase-8 activity and relatively un-

changed caspase-9 activity indicate that apoptosis occurred through activation of the extrinsic pathway. We also found that bcl-xl levels fell with EphB4 knockdown, whereas no effect was observed in the levels of bcl-2 and Mcl-1. Similar to our results, it has been reported previously that bcl-xl is expressed at high levels in mesothelioma, whereas the expression of bcl-2 and Mcl-1 is very low (39). Reduction in bcl-xl as observed in our studies may thus have significant effect in mesothelioma cell viability. Further work is needed to define the role of bcl-xl, if any, in EphB4-mediated cell survival signal in mesothelioma.

EphB4 activation allows interaction with a variety of cytoplasmic signaling proteins. Many of these proteins have been implicated in regulating cell morphology, cytoskeletal organization, attachment, and motility (40–42). Activation of EphB4 leads to downstream signaling via PI3K; in agreement with which, we show a decline in the activation status of the downstream molecule Akt following EphB4 knockdown. To our knowledge, this is the first report that shows a role for EphB4 in regulation of Akt with a potential role in tumor cell survival. It has been shown that EphB4 receptor signaling mediates endothelial cell migration (40). EphB4/NeuT double transgenic animals develop metastatic tumors in contrast to NeuT transgenic animals, which form tumors only in the mammary gland without metastases (43). We found that the inhibition of EphB4 significantly inhibits migration and invasion. MMPs play a significant role in tumor invasion and angiogenesis. MMP-2 is overexpressed in mesothelioma (44). In a recent study, it has been shown that MMP-2 and MMP-9 are activated and function in endothelial migration on activation of EphB4 (40). Our findings that EphB4 regulates MMP-2 but not MMP-9 in mesothelioma may suggest a potential role for EphB4 in regulation of tumor cell migration and invasion. The inhibition

of EphB4, apart from directly inhibiting tumor survival, may thus prevent tumor progression as well as metastasis.

To further elucidate the possible therapeutic role of targeting EphB4 in the treatment of mesothelioma, we conducted *in vivo* efficacy studies. EphB4-specific oligodeoxynucleotide significantly inhibited tumor growth, reduced tumor cell proliferation, and induced apoptosis in comparison with control or scrambled oligodeoxynucleotide treatments. Tumor growth inhibition can result from a direct effect on tumor cells as observed *in vitro*. In addition, EphB4 on tumor cells bind EphrinB2 on tumor vessels and thereby induce an angiogenic response (45). Down-regulation of EphB4 may thus have an additional and independent effect on tumor vasculature as seen in our study. Hence, EphB4 knockdown has a dual inhibitory effect on tumor growth by directly inhibiting tumor cell survival as well as inhibiting tumor-associated angiogenesis.

In conclusion, we show the expression of the RTK EphB4 in malignant mesothelioma. Receptor overexpression may favor uncontrolled cell growth, migration, and tumor progression. EphB4 targeting could serve as novel therapy for patients with mesothelioma.

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