Overexpression of EPH Receptor B2 in Malignant Mesothelioma Correlates with Oncogenic Behavior

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Introduction: Malignant pleural mesothelioma (MM) is an aggressive asbestos-associated malignancy with limited therapeutic options. This study describes the overexpression of Ephrin B2 receptor (EPHB2) in MM cell lines and tumors, and the effect of its manipulation on proliferative and invasive qualities of the disease.

Methods: Using expression arrays, we investigated EPHB2 in MM tumors compared with normal mesothelium. EPHB2 and downstream target expression were evaluated using reverse-transcriptase polymerase chain reaction and immunoblotting methods. The biological significance of EPHB2 in MM was evaluated using in vitro functional assays with and without targeting by EPHB2-short hairpin RNA or blocking peptide in two mesothelioma cell lines, HP-1 and H2595.

Results: EPHB2 is overexpressed in all MM cell lines, but not in benign mesothelial cells, and is significantly elevated in MM tumor tissue compared with matched normal peritoneum. Targeted knockdown of EPHB2 in HP-1 and H2595 cell lines reduced its expression and that of EPHB2 downstream targets such as matrix metalloproteinase (MMP-2) and vascular endothelial growth factor, whereas caspase 2 and caspase 8 had increased expression. Inhibition of EPHB2 resulted in a significant decrease in scratch closure (1.25-fold–1.8-fold), proliferation (1.5-fold), and invasion (1.7-fold–1.8-fold) compared with the controls. Most notably, however, EPHB2 silencing resulted in a significant increase in apoptotic proteins and activity.

Conclusion: EPHB2 seems to play an important role in MM pathogenesis and these findings indicate that EPHB2 could serve as a potential novel therapeutic target for treatment of the disease.

Key Words: Mesothelioma, Ephrin receptor B2, Invasion, Proliferation, Apoptosis.

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Malignant mesothelioma (MM) is a rare asbestos-induced, aggressive neoplasm that most often arises from the serous surface of the pleural and peritoneal cavity, and is associated with a poor prognosis.[1,2] It is relatively rare in the United States, about 2500 to 3000 cases are diagnosed each year and the majority are diagnosed in late stages when the disease shows a high resistance to all types of conventional treatments such as surgery, chemotherapy, and radiotherapy.[3] Despite advances in the treatment of MM, the median survival remains 12 months from the time of diagnosis. Increased understanding of the molecular basis for the diverse signaling pathways involved in cancer progression should promote the discovery of novel biomarkers for early diagnosis and potentially lead to more effective therapeutic tools for the disease.

Ephrin (EPH) receptors and their ligands constitute the largest subfamily of receptor tyrosine kinases (RTKs) and are critical to a wide range of cellular processes during embryonic development, cell aggregation and migration, segmentation, neural development, angiogenesis, vascular hierarchical remodeling, and cytoskeleton alterations.[4–7] The EPH family members are subdivided by sequence identity into A class and B class according to their binding capacities with corresponding ligands.[8]

EPH-RTK signaling regulates cancer initiation and metastatic progression through multiple mechanisms, and signaling events activated by their corresponding ligands may be bidirectional, forward, or a reverse manner.[9] In fact, the literature on tumor-cell autonomous effects of EPH receptors demonstrates a dual role for tumor suppression and tumor promotion.[10–14] The complex nature of EPH receptor signaling and crosstalk with other RTKs presents a unique challenge as well as opportunity to develop novel strategies for targeting malignancy. EPH receptors and ligand overexpression in a variety of human cancers correlates with cancer progression,[4,9] with some studies implicating effects on the angiogenic pathways[15–20] and apoptosis.[21,22] In the current study, we examined the expression of the Ephrin B2 receptor (EPHB2) in tumor and corresponding normal peritoneum, as well as in benign and MM cell lines. We report that silencing the EPHB2 gene expression in MM cell lines decreased its protein expression, resulted in a significant decrease in cell proliferation, migration, colony formation, as well as an increase in apoptosis-related caspase-8 activity. Further, the use of an EPHB2-blocking peptide altered proliferation and cell invasion of mesothelioma cells.

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MATERIALS AND METHODS

Cell Lines

MM cell lines HP1, HP3, H2373, H2452, H2591, H2595, H2596, and H2461, tert immortalized mesothelial cell line LP9,[23] primary mesothelial cell culture NYU590.2 (passage 3), and simian virus 40 (SV40)-transformed mesothelial cell line Met5A were grown in 1X Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS; Invitrogen) and pencillin/streptomycin (Invitrogen, Carlsbad, CA). Human mesothelial cells (NYU590.2) were surgically isolated from a noncancer patient.

Clinical Specimens

Thirty-four matched MM tumors and normal peritonea from patients undergoing extrapleural pneumonectomy were used for differential gene-expression analysis of EPHB2 using human genome 1.0 ST (HG 1.0ST) microarray chips (Affymetrix Inc., Santa Clara, CA). Expression data was validated by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analysis using RNA and protein lysate from seven matched tumor/normal tissue pairs as previously described.[24]

RNA Extraction

Cell lines and tissue RNA were extracted using RNEasy Mini Kit from Qiagen (QIAGEN Inc., Valencia, CA) following manufacturer's protocol.

Semiquantitative RT-PCR

Semiquantitative RT-PCR analysis was performed using SuperScript III One-Step PCR Kit (Invitrogen). For the detection of EPHB2, gene-specific primers, 5′-CTTCGAGGCCGTTGAGAAT-3′ (forward) and 5′-GAGCCACAGCTCTTGCAGAT-3′ (reverse) spanning at least two exons were designed based on National Center for Biotechnology Information (NCBI) gene bank sequences (NM_017449.3) annealing at 58°C for 30 cycles. The PCR products were subjected to electrophoresis in 1.5% agarose gels stained with ethidium bromide.

Quantitative Real-Time Polymerase Chain Reaction Analysis

The mRNA was quantitatively measured by using StepOne real-time PCR System (Applied Biosystems, Carlsbad, CA). Superscript III First Strand Synthesis Super Mix (Invitrogen) was used to generate first strand cDNA. Samples were assayed in 20-µl reaction mixture containing 50 ng of cDNA, 200 nM of gene-specific primers (Table 1), 2X SYBR Green Master Mix (Life Technologies, Grand Island, NY) and molecular grade H_2O. The amplifications were performed for 40 cycles at 58°C for 1 minute. Samples were run in duplicates including negative controls. Relative quantification ($2^{-\Delta\Delta CT}$ method) was performed to determine the relative quantities. Peptidylproplyl isomerase A gene was used as a normalization control in both semi- and quantitavie real time polymerase chain reaction (qRT-PCR) analysis.

Antibodies and Blocking Peptides

Actin (sc-1616), EPHB2 (sc-130752), MMP9 (sc-13594), VEGF (sc-7269), caspase 1 (sc-515), caspase 8 (sc-6136), BIRC5 (sc-17779), BCL2L1 (sc-7195) and EPHB2-blocking peptide (sc-1763P) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) to be used for the Western blotting experiments (see below).

shRNA Plasmids and Blocking Peptide Treatments

Two independent EPHB2-shRNA constructs containing a short-hairpin target sequence (NCBI Ref ID: NM_004442.6) of 19mers, 5′-ACCTCGTCTACAACATCAT-3' (HSH004939-1-LvH1) and 5′-ACCTCGTCTACAACATCAT-3′ (HSH004939-2-LvH1), and a scrambled control (CSHCTR001-LvH1) plasmids were purchased from GeneCopoeia (Rockville, MD). MM cell lines H2595, HP-1, and normal mesothelial LP9 cells were transfected with 4 µg of either scrambled control or EPHB2-shRNA plasmids in a 10-cm dish for 48 hours using Lipofectamine 2000 (Invitrogen) as recommended. Forty-eight hours posttransfection, cells were used for Western blot analysis, immunofluorescence, and function behavior studies as described. For blocking experiments, H2595 and HP-1 cells were treated with 0.2 and 2 µg/ml of EPHB2-blocking peptide.

TABLE 1. Quantitative Real-Time-Polymerase Chain Reaction

Gene	NCBI Ref Seq No.	Forward Primer	Reverse Primer	Annealing Temp (°C)	Product Size (bp)
EPHB2	NM_017449.3	TGGCTACGATGAGAACATGAA	CTTGGTCCGTAGCCAGTTGT	58	88
Caspase1	NG_029124.1	TGCCAAATTTGCATCACATA	TTCCAGATGTTTGATCTGCTG	58	85
Caspase 8	NM_001228.4	CTACATTCCGCAAAGGAAGC	GGACAGATTGCTTTCCTCCA	58	97
BCL2L1	NG_029002.1	TGGAAGAGAACAGGACTGAGG	AGGATGGGTTGCCATTGAT	58	81
BIRC5	NM_001168.2	GAGGCTGGCTTCATCCACT	CAGCTCCTTGAAGCAGAAGA	58	75
MMP-2	NG_008989.1	CCCAAAACGGACAAAGAGTT	ACAGGTTGCAGCTCTCCTTG	58	76
VEGF	NM_001171623.1	AAGGAGGAGGGCAGAATCAT	CTCGATTGGATGGCAGTAGC	58	77
PPIA	NM_021130.3	GCTCTGAGCACTGGAGAGAAA	GAAGTCACCACCCTGACACA	58	90

Gene-specific primers and optimized conditions.
EPHB2, ephrin B2 receptor, MMP-2, matrix metalloproteinase; VEGF, vascular endothelial growth factor; PPIA, peptidylproplyl isomerase.

Western Blot Analysis

Total cell proteins were prepared by lysing the cells in 10 mM/liter Tris (pH 7.5), 1 mM/liter EDTA, 150 mM /liter NaCl, 1% Triton X-100, 1 mM/liter DTT, 10% glycerol for half-hour on ice. The lysates were centrifuged and cleared at 13K, and total protein was estimated by bicinchoninic acid (BCA) Protein Assay Kit (Thermoscientific, Rockford, IL). Twenty-five micrograms of samples were separated on 8% to 16% Tris-Glycine gel (Invitrogen, Green Island, NY) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). The membranes were blocked in 5% nonfat dry milk for 1 hour at room temperature followed by overnight incubation in primary antibody at 4°C and then incubated in secondary antibody conjugated with horseradish peroxidase for 1 hour at room temperature. SuperSignal West Pico Chemiluminescent Substrate (Thermoscientific, Rockford, IL) was used to develop the membrane. To ensure equal loading and transfer of proteins, membranes were stripped and reprobed with Actin antibody.

Immunoflourescence

Scrambled control and EPHB2-shRNA plasmids transfected mesothelioma cells, H2595 and HP-1 were cultured on Lab-Tek II 4-well chamber slides (Thermoscientific) 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 primary antibodies to EPHB2 (1:100) in blocking buffer at 4°C overnight. After washing thrice, the slides were incubated with the fluorescein-conjugated secondary antibody (Molecular Probes, Eugene, OR). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole, washed extensively with PBS, and mounted with Vectashield antifade mounting solution (Vector Laboratories, Burlingame, CA). Images were obtained using a Nikon Eclipse 90i fluorescence microscope (Nikon Instruments Inc., Melville, NY) using NIS Elements digital imaging software (Nikon Instruments, Inc.).

Matrigel Invasion Assay

The BD Biocoat Matrigel invasion chambers (BD Sciences, Franklin Lakes, NJ) were used according to the manufacturer's protocol. We transferred 5×10^4 posttransfected cells onto a 8 μM pore-size polycarbonate membrane inserts coated with a thin layer Matrigel basement membrane matrix without phenol-red (BD Sciences). For peptide treatment, the cells were first seeded on coated inserts and then incubated with the blocking peptide for 48 hours at 37°C and 5% CO_2. The inserts were removed and noninvading cells on the upper surface were removed with a cotton swab. The cells that migrated through the Matrigel (Franklin Lakes, NJ) to the lower surface were fixed and stained with Giemsa solution and 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.

Cell-Proliferation Assay

H2595 and HP-1 cells transfected with scrambled control, and EPHB2-shRNA plasmids were seeded onto a 96-well plate at a density of 4×10^4 cells per well in 100 μl of DMEM/10% FBS and incubated at 37°C for 48 hours. For peptide treatment, cells were seeded and then incubated in blocking peptide. Twenty microliters of Cell-Titer-Blue reagent (Promega, Madison, WI) was added to each well and incubated for 1 hour. Optical density was measured at 560 of 590 λ using the Universal Reader Victor^3 (PerkinElmer, Waltham, MA).

Scratch Closure Assay

We seeded 2×10^5 transfected cells in 6-well plates. After 24 hours, a single scratch was made through the bottom of the plate using a 1-ml sterile pipette tip. Plates were washed with PBS to remove floating cells. The undersides of plates were marked and the scratch width was measured at three locations. Plates were incubated for 24 hours and scratch width measured at the same three points in reference to marks. Closure was calculated as a percent of initial scratch width.

Soft-Agar Colony Formation

Plates with base agars were made before 30 minutes of cell seeding with equal volumes of 1% agarose and 2 × DMEM/10% FBS, both at 40°. A 1.5 ml of warm base agar was poured into 6-well plates and allowed to polymerize. The top agar was produced by combing equal volumes 5×10^3 transfected cells/ml in 1×DMEM/10% FBS with warm 0.7% agarose. Two milliters of the agarose cell mixture was placed on top of each base coat. Plates were incubated at 37°C, 5% CO_2 for 14 to 21 days. Colonies were stained with 0.5 ml of 0.005% Crystal violet for more than 1 hour and counted under a dissecting microscope.

Caspase-8 Activity Assay

Caspase-8 activity assay was performed as described by the manufacturer (Genscript, Piscatway, NJ) to measure apoptosis. Then 3×10^5 posttransfected cells were collected and washed twice with 1× PBS. The cells were incubated in lysis buffer for 60 minutes on ice and centrifuged at 10,000 rpm at 4°C for 10 minutes. Clear lysate supernatant containing 200 μg of protein was assayed using caspase-8 substrate. The caspase-8 activity was measured at 405 nM using a spectrophotometer.

Statistical Analysis

The EPHB2-dependent changes from all functional assays were analyzed for statistically significant differences by standard Student's *t* test, with *p* value less than 0.05 considered significant. All statistical analysis were performed with the use of SigmaPlot, Version 8 (San Jose, CA)

RESULTS

EPHB2 Is Overexpressed in MM Tumors

Gene-expression profiles of 34 matched MPM tumors and normal peritoneum were established using Affymetrix HG 1.0ST microarrays followed by analysis of microarray data. We found significant (p = 2.39E-05) overexpression

of EPHB2 by 3.75-fold change in tumors compared with its normal peritoneum (Fig. 1*A*). A similar pattern of expression is seen on U133plus2.0 arrays (see supplementary data 3, http://links.lww.com/JTO/A449). In reviewing EPHB2 data in GEOPROFILES, we found confirmatory data using the U133 Affymetrix platform, which demonstrated a relative expression of normal pleura (n = 5) to be 32 relative units compared with 71 for 42 mesothelioma tumors (http://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GDS1220:209589_s_at).

We validated this data by qRT-PCR and Western blot analysis in seven matched tumor/normal tissue pairs from the same set that were used for the microarray analysis. Quantitative RT-PCR analysis showed a significant elevation in EPHB2 mRNA in tumors compared with matched normal peritoneum (see supplementary data, Figure 1, Supplemental Digital Content 1, http://links.lww.com/JTO/A447). The qRT-PCR results correlated well with the elevated levels of EPHB2 protein in tumors seen by Western blot analysis (Fig. 1*B*).

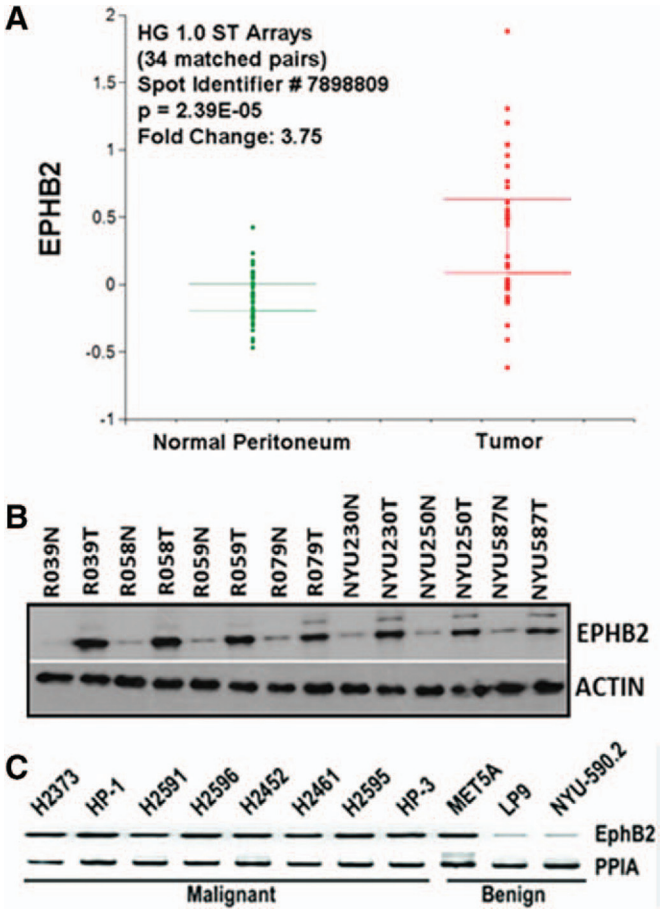

FIGURE 1. EPHB2 is overexpressed in MM tumors and cell lines. *A*, Affymetrix HG 1.0ST microarrays reveals a 3.75-fold (p = 2.39E-05) increase in EPHB2 gene-expression levels in tumors compared with matched normal peritoneum. *B*, Western blot analysis of tissue lysates also demonstrates overexpression of EPHB2 in clinical MM tumor specimens compared with matched normal peritoneum. *C*, Semiquantitative RT-PCR analysis reveals elevated levels of EPHB2 in MM cell lines compared with benign cell lines. PPIA, peptidylproplyl isomerase; EPHB2, ephrin B2 receptor; MM, malignant mesothelioma; NYU, New York University.

EPH B2 Is Overexpressed in Mesothelioma Cell Lines

The expression of EpEPHB2 was evaluated in eight mesothelioma cell lines and two benign mesothelial cell lines by semiquantitative RT-PCR method. We noted an increase in the levels of EPHB2 mRNA expression in MM cell lines compared with benign mesothelial cells (Fig. 1*C*). These results were validated by qRT-PCR (see supplementary data, Figure 2, Supplemental Digital Content 2, http://links.lww.com/JTO/A448).

Knockdown of EPHB2 by Its shRNA Decreased Its mRNA and Protein with an Increase in Apoptotic Cells

After validating overexpression of EPHB2 in MM tumor tissue and cell lines, we studied its functional behavior by knocking it down with a gene-specific short-hairpin sequence consisting of 19 bases. Two MM cell lines, H2595, HP-1, and one benign LP9 cell line were successfully transfected with EPHB2-shRNA and scrambled control plasmids for 48 hours. Posttransfected cells were evaluated for EPHB2 mRNA and protein expression by qRT-PCR and Western blot analysis. Both H2595 and HP-1 cells showed 80% to 90% knockdown of its mRNA expression (Fig. 3*A*). Western blot analysis reflected a 90% reduction in its protein levels compared with treated controls (Fig. 2*A*). The reduction in EPHB2 expression was also confirmed by immunofluorescence in both of the mesothelioma cell lines cultured on chamber slides (Fig. 2*B*).

Further analyses of downstream genes by qRT-PCR revealed that silencing EPHB2 with shRNA in H2595 and HP-1 cells resulted in significant decreases in the mRNA levels of matrix metalloproteinase (MMP-2), VEGF, and antiapoptotic genes BCL2 and BIRC5 (survivin). Moreover, up-regulation of apoptotic genes such as caspase 1 and caspase 8 (Fig. 3*A*) was recorded as well, as a 30% to 40% increase in apoptotic cells in HP-1 and H2595 MM cells compared with scrambled control (Fig. 3*C*). Similar changes were noticed in the protein of these downstream targets by Western blot analysis (Fig. 3*B*). However, no changes were recorded with EPHB2 knockdown in LP9 cells.

EPHB2 Suppression Is Involved in Decreased Cell Proliferation, Migration, Invasion, and Colony Formation

To study the significance of EPHB2 expression on the biological behavior of MM we performed a series of in vitro functional assays. Our results demonstrate that targeting EPHB2 in H2595 and HP-1 cells with shRNA resulted in a significant decrease in the tumorigenic properties but has no effect in LP9 benign mesothelial cells. In cell-proliferation assay, there was a 1.4-fold to 2.0-fold ($p < 0.0015$) decrease in cell density in both the MM cell lines, H2595 and HP-1, with EPHB2-shRNA compared with scrambled controls (Fig. 4*A*).

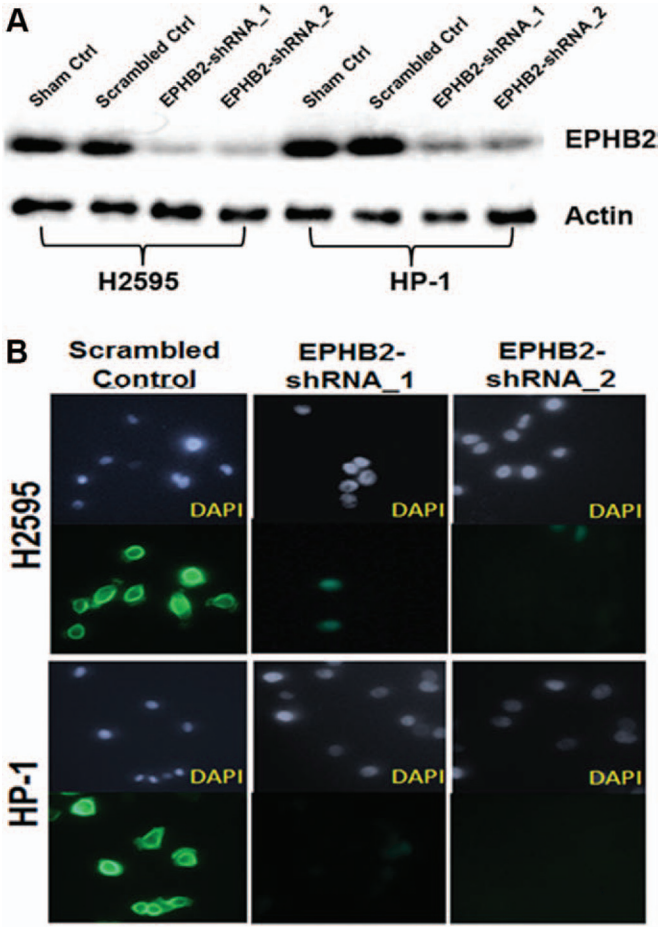

FIGURE 2. Targeted knockdown of EPHB2 with shRNA results in a significant decrease in EPHB2 protein expression (*A*) in H2595 and HP-1 cells. *B*, Immunofluorescence studies demonstrate decreased EPHB2 expression in H2595 and HP-1 MM cells after expression of its specific shRNA. EPHB2, ephrin B2 receptor.

Cells transfected with EPHB2-shRNA plasmids had significant decreases in horizontal migration in a scratch closure assay. In H2595 cells, the scratch closure was reduced by 29% ($p < 0.0005$) and by 25% ($p < 0.0005$) in HP-1 cells compared with treated scrambled controls (Fig. 4*B*).

Silencing EPHB2 expression in H2595 and HP-1 cell lines significantly decreased the ability of the cells to invade through matrigel compared with treated scrambled control cells used in the studies. Both H2595 and HP-1 cells showed 2.3-fold to 2.7-fold ($p < 2.00E-05$) and 1.5-fold to 1.7-fold ($p < 2.00E-05$) decrease in cell invasion (Fig. 4*C*). In a soft agar colony formation assay, EPHB2 knockdown exhibited a significant decrease in the size and number of colonies in both the cell lines at 21 days (Fig. 4*D*).

Blocking EPHB2 Expression by Its Peptide Showed a Significant Reduction in Cell Proliferation and Cell Invasion

In an effort to further validate the functional aspects of EPHB2 expression in MM, both H2595 and HP-1 cells were treated with immunoglobulin control or EPHB2-blocking peptide using concentration of 0.2 μg/ml or 2 μg/ml. After 24 hours of treatment, there was a significant decrease in the cell proliferation compared with the treated controls. Cells receiving 0.2 μg/ml of the blocking peptide had a 2.1-fold ($p = 0.031$) and 2.56-fold ($p = 0.003$) decrease in cell proliferation. At 2 μg/ml concentrations, there was a 2.5-fold decrease in cell proliferation in H2595 ($p = 0.0002$) and 2.8-fold decrease in HP-1 cells ($p = 0.003$) (Fig. 5*A*).

Similarly, in matrigel invasion assays, at 24 hours, 2 μg/ml of blocking peptide revealed a 2.0-fold decrease in cell invasion for H2595 cells ($p = 0.0002$) and a 1.6-fold decrease in HP-1 cells ($p = 3.41E-05$) (Fig. 5*B*). All the fold changes were calculated in comparison with treated controls.

DISCUSSION

Despite progress in identifying the molecular changes in MM, no targeted therapy has yet proven consistently effective in MM. EPH receptors and their ligands, EPHs, form the largest subfamily of tyrosine kinases and were initially identified as neuronal path-finding molecules, which guide migrating cells to specific tissue targets that are essential in embryonic development, vascular cell assembly, and angiogenesis.[6] Their functions have mainly been studied with respect to cell-to-cell interaction, migration, motility, and angiogenesis. Increasing evidence points to the involvement of EPH receptors and EPH ligands in cancer progression, although their exact role is still unknown; however, surprisingly, the receptors and ligands have both tumor promoter and suppressor functions in different cellular contexts.[25–29] Overexpression of some members of EPH receptor family has an important role in the development and progression of various cancers. In particular, EPHB2 overexpression is frequently associated with human invasive cancers.

This report details that that EPHB2 is overexpressed in MM cell lines as well as in tumor samples. MM is characterized by extensive local growth and invasion of intrathoracic organs. This kind of tumor growth suggests the degradation of the extracellular matrix and basement membrane by proteases such as MMPs in the course of the disease. It has been shown that stromal modifications, such as proteolysis of the extracellular matrix and basement membrane, are mediated by MMPs. MMPs are a family of zinc-dependent enzymes, which are implicated in the growth of primary and secondary tumors.[30] MMPs play a significant role in tumor invasion and angiogenesis. MMPs, in particular gelatinase A (MMP-2) and gelatinase B (MMP-9) are overexpressed in MM.[31–33] Our findings indicate that EPHB2 may modulate expression of MMP-2.

Caspases, a family of cysteine proteases, play a critical role in the execution of apoptosis, an evolutionary conserved ATP-dependent type of programmed cellular death process.[34–38] They are synthesized as proenzymes that are processed by self-proteolysis and/or cleavage by other protease members to their active forms in cells under process of apoptosis. Apoptosis can be triggered by either intrinsic or extrinsic death signals and is regulated by two gene families, Bcl2 and IAP. The Bcl-2 protooncogene can block apoptotic cell death

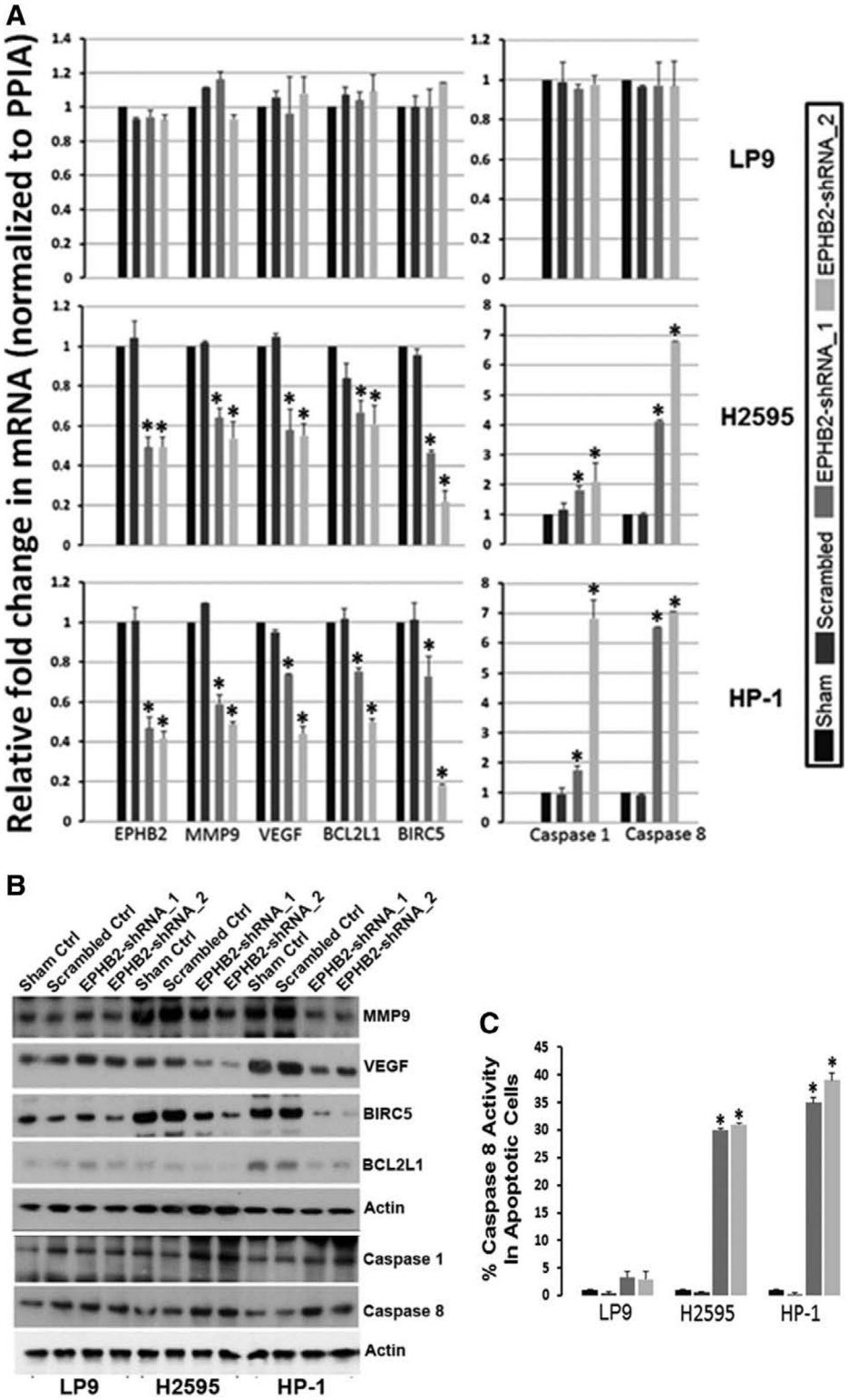

FIGURE 3. *A*, qRT-PCR and Western blot analysis of EPHB2 downstream genes: Targeted knockdown of EPHB2 expression by its shRNA causes an increased expression of caspase 1 and caspase 8 mRNA (*A*) and protein (*B*). A decreased expression of BCL2L1, BIRC5, matrix metalloproteinase, and vascular endothelial growth factor mRNA (*A*) and protein (*B*) is noticed in H2595 and HP-1 cells. *C*, Caspase-8 activity: A significant increase in the caspase-8 activity in apoptotic cells is seen after forced expression of EPHB2-shRNA in H2595 and HP-1 cells compared with scrambled control. Peptidylproplyl isomerase A gene was used as a normalization control in both semi and qRT-PCR analysis. EPHB2, ephrin B2 receptor; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; EPHB2, ephrin B2 receptor.

either by regulation of free radical generation[39] or by blocking cyctochrome C release.[40] BIRC5/Survivin is a member of the inhibitor of apoptosis protein (IAP) family and also acts as an important antagonist of apoptosis and has been reported to be elevated in MM.[41,42] Manipulating members of EPH receptor family may induce apoptosis as it has been shown that silencing EPH receptors induces apoptosis in MM.[21,22,42] In our study, we have shown that knockdown of EPHB2 essentially is apoptosis inducing, in that the expression of caspase 1 and caspase 8 become significantly elevated and the levels

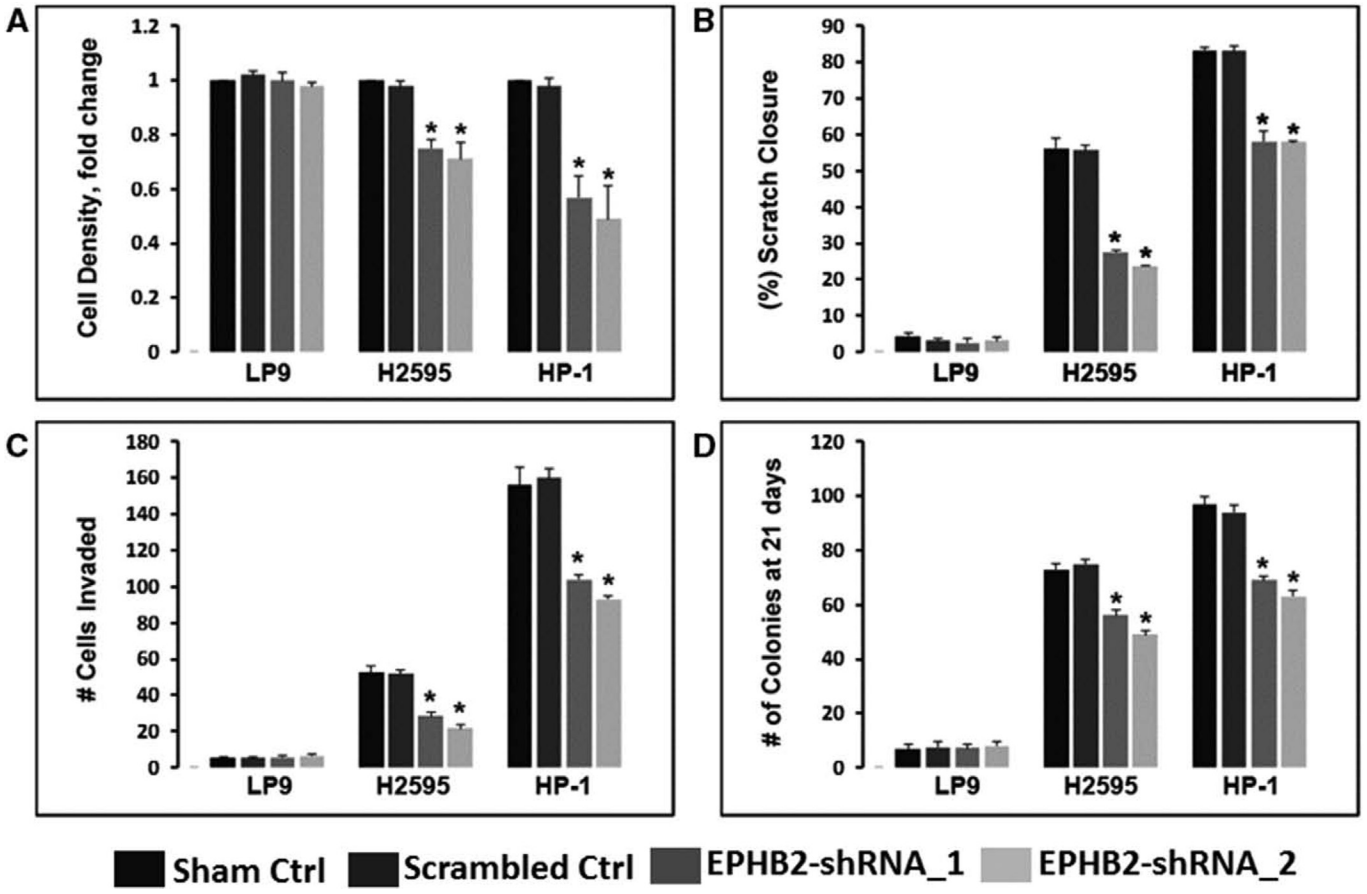

FIGURE 4. Biological significance of EPHB2 in MM is demonstrated in in vitro functional assays after suppression of EPHB2 expressions by its shRNA. *A*, Cell-proliferation assay: a significant decrease in cell proliferation with a fold change of 1.4-fold to 2.0-fold ($p < 0.001$) was noticed in both H2595 and HP-1 cells. *B*, Scratch closure assay: a reduction in horizontal migration by 29% ($p < 0.0005$) in H2595 and 25% ($p < 0.0005$) in HP-1 is noticed. *C*, Cell invasion is significantly decreased in H2595 by 2.3-fold to 2.7-fold ($p < 2.00E-05$) and in HP-1 by 1.5-fold to 1.7 fold ($p < 2.00E-05$) compared with scrambled controls. *D*, Colony formation: significant decrease in the size and number of colonies was noted in both H2595 and HP-1 MM cells. However, no significant changes were noticed in LP9 cells. EPHB2, ephrin B2 receptor, MM, malignant mesothelioma.

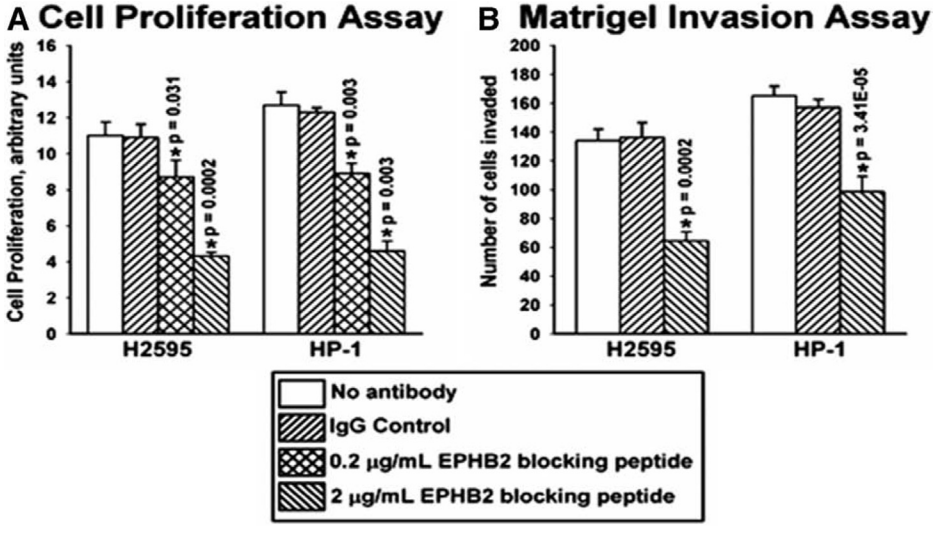

FIGURE 5. Blocking peptide and antibody experiments in H2595 and HP-1 cells show a significant decrease in cell proliferation (*A*) and invasion (*B*). EPHB2, ephrin B2 receptor.

of Bcl-2 and survivin are decreased. Hence, it is theoretically possible to modulate MM viability through manipulations of EPHB2.

EPH receptors and EPH ligands play an important role in the metastatic process not only by affecting migration, apoptosis, and, but also through angiogenesis.[43,44] It has been shown that some growth factors play an important role in the growth and metastasis of MM such as platelet-derived growth factor (PDGF)-B, insulin-like growth factor (IGF)-1, transforming growth factor (TGF)-a, interleukin (IL-8), VEGF, and VEGF-C[45–48] of which IL-8 and VEGF are potent angiogenic factors.[49,50] Elevated levels of EPHB2 in MM may regulate VEGF expression, and our results demonstrate a decrease in VEGF expression with reduced EPH receptor B2 activity.

In conclusion, EPHB2 is overexpressed in MM, and it inhibition has profound in vitro effects on the invasive aspects of the disease as well as on apoptosis control. Therefore, targeting EPHB2 might provide a novel therapy to improve the prognosis in people suffering from MM. Further investigation in vitro using specific inhibitors of EPHB2 is required to determine the importance of EPHB2 and its interactions with other members of the receptor kinases and their ligands to prove its role as a marker of progression and metastasis for MM.

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