**Antibodies and other reagents**

Bovine serum albumin, gelatin, and MTT were obtained from Sigma-Aldrich. IGF-I was purchased from R&D Systems and Amicon Ultra-4 concentrator units were purchased from Millipore Co. Anti-bFGF, anti–Sp-1, anti–Egr-1, anti-MEK1/2, anti-ERK1/2, and anti–β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-IGFBP-3 and anti-CD31 antibodies were purchased from Diagnostic Systems Laboratories, Inc. and BD Biosciences, respectively. IGFBP-3 and nonspecific control siRNA were purchased from Dharmacon. Ad-BP3 and Ad-EV were amplified as previously described. rBP3 was obtained from Insmed. Constructs for the *Egr-1* promoter analysis were generous gifts from Dr. David M. Cohen (Oregon Health Sciences University, Portland, Oregon).

**Cell proliferation assay**

To test the effects of either IGFBP-3 expression or CM from the NSCLC cell cultures on cell proliferation and survival, HUVECs (1 × 10⁴) were incubated in endothelial growth medium (EGM; Lonza Bioscience) with CM collected from rBP3 (10 μg/ml)- or Ad-BP3–treated NSCLC cells. After 24 hr, the media were replenished with fresh EGM or a 1:1 mixture of EBM (Cambrex BioScience) and the CM. Either EBM or a mixture of EBM and serum-free RPMI-1640 was used for the negative controls. After 3 days of incubation, the cells were subjected to the MTT assay. Six replicate wells were used for each analysis, and at least three independent experiments were performed.

**Chick aortic ring arch assays**

The aortic ring arches of 14-day-old chick embryos were dissected and placed on a 10 μl Matrigel drop in 48-well plates and EBM was added. Average sprouting was measured with Axiovision 4.3 software (Carl Zeiss) after the plates were photographed under a stereomicroscope (Carl Zeiss). Each condition was replicated in six wells, and the experiment was repeated three times. To determine the effects of IGFBP-3 on the stimulation of angiogenesis in NSCLC cells *in vitro*, CM collected from H1299 cells infected with either Ad-BP3 or Ad-EV were added to EBM. HUVECs were incubated with EBM in the presence or absence of CM for 48 hr to allow microvessel sprouting from the adventitial layer.

**Northern blot hybridization**

Twenty micrograms of total RNA from H460 cells transfected with Ad-BP3 or Ad-EV, treated with rhBP3 protein were separated by electrophoresis in denaturing agarose gels and transferred to a Zeta-probe membrane (Bio-Rad Laboratories). Membranes were hybridized with [³²P]-dCTP random-primed each gene cDNA probe, washed, and visualized by autoradiography. Ethidium bromide staining was used to ensure equal RNA loading. GAPDH mRNA and 28s RNA were monitored as an internal controls.

**Construction of Egr-1 promoter mutants and luciferase reporter assay**

The constructs of *Egr-1* promoter mutants were generated by site-directed mutagenesis of wild-type *Egr-1* promoter construct (*Egr-C*) using Pfu polymerase and mutagenic oligonucleotide primers designed to introduce each specific multi-base mutation. The sequences of the oligonucleotides used for mutation of the SRE sites 3, 4, and 5 within the *Egr-1* promoter are shown below, with the mutated sequences in lowercase: for the SRE-3 mutation, 5′-TGGGCAGCCCTTAa gTGGA GTGGC CCAAT A-3′; for the SRE-4 mutation, 5′-CGGAA CAGAC CTTAT TaatG CAGCG CCTTA TATGG-3′; for the combined mutation of SRE-3 and -4, 5′-CGGAA CAGAC CTTAT TaatG CAGCG CCTTA agTGG-3′; for the SRE-5 mutation, 5′-CCGGA AACGC CATAT gAaGA GCAGG AAGGA TCC-3′. The DNA sequence of each mutant
clone was confirmed by DNA sequencing. For the luciferase reporter assays H460 cells were harvested 2 days after transfection with the appropriate mutant Egr-1–promoter luciferase reporter plasmid and β-gal control vector, pCH110, and the cell extracts were assayed for luciferase activity as described elsewhere. Cell extracts were assayed for luciferase activity using luciferin (Sigma-Aldrich) as the substrate and normalized to β-gal activities. Luciferase activities were expressed as the mean ± SD of measurements made from three identical wells.

**Chromatin immunoprecipitation**

The sequences of primers for amplification of the SRE elements and exon 1 for ChIP analysis are as follows: SRE primers, 5′-AGGGA GCGAG GGAGC AAC-3′ and 5′-GCTTC GGGGA AGCCT AGAG-3′; exon 1 primers, 5′-CTGCA CGCTT CTCAG TGTTC-3′ and 5′-TCATC TCCTC CAGCT TAGGG-3′. The PCR conditions were as follows: 95°C for 15 min to activate the HotStar Taq polymerase followed by 94°C for 2 min, 21 to 23 cycles of 94°C for 30 sec, 60°C for 30 sec to anneal, and 72°C for 1 min to elongate, and finally 72°C for 3 min.

**Quantitative RT-PCR**

The primer sequences for quantitative RT-PCR analysis are as follows: for IGFBP-3 (GenBank accession number, NM_000598), 5′-GAAGG GCGAC ACTGC TTTTT C-3′ (sense) and 5′-CCAGC TCCAG GAAAT GCTAG-3′ (antisense); for Egr-1 (GenBank accession number, NM_001964), 5′-AAAGT TTGCC AGGAG CGATG-3′ (sense) and 5′-CAGGG GATGG GTATG AGGTG-3′ (antisense); for bFGF (GenBank accession number, NM_002006), 5′-TCACC AGCCT GCCCG CCTTG C-3′(sense) and 5′-CAGTT CGTTT CAGTG CCACA T-3′ (antisense); for Sp-1 (GenBank accession number, NM_138473), 5′-CAAGC CCAAA CAATC ACCTT-3′ (sense) and 5′-TCCAC CTGCT GCCCG CCTTG C-3′(sense); for PDGF-a (GenBank accession number, NM_008808.3), 5′-GCGGC TACAT GAGA GAAGT A-3′ (sense) and 5′-GGCA GAGA GAAGT A-3′ (antisense); for PDGF-b (GenBank accession number, NM_011057.3), 5′-GCACT GTAGG GTATG AGGTG-3′ (antisense); for PDGF-c (GenBank accession number, NM_019971.2), 5′-TGCA GCGAG GGAGC AAC-3′ (sense) and 5′-TGACT CCTCT TGGTG CCTCT-3′ (antisense); and for GAPDH (GenBank accession number, NM_002046), 5′-GGTG AAA GTG CGG TGT GAA ATT T-3′ (sense) and 5′-AAT GCC AAA GTT GTC ATG GAT GAC C-3′ (antisense).

**Transfection of IGFBP3-containing plasmids and siRNAs**

H460 (1.5 × 10⁶ cells/100-mm dish) cells were seeded and transfected with either pIGFBP3-ggg (5 µg) or pcDNA 3.1(5 µg) using the lipofectamin reagent (Life Technologies). For the luciferase reporter assay, luciferase constructs (0.5 µg) were cotransfected into H460 cells with 0.01 µg of the β-gal control vector, pCH110. The cells were transfected in serum-free media for 6 hr and then were switched to a medium containing 10% FBS. Then, the cells were subjected to either luciferase reporter assays or western blot analysis. For the transfection of siRNA, H460 cells (1 × 10⁵ cells/well) were plated and transfected with 100 mM siRNA using oligofectamine (Invitrogen). After 2 days of incubation, cells were lysed for immunoblot analysis.

**Northern blot hybridization**

Twenty micrograms of total RNA from H460 cells transfected with Ad-BP3 or Ad-EV, or H460 cells treated with rBP3 were separated by electrophoresis in denaturing agarose gels and transferred to a Zeta-probe membrane (Bio-Rad Laboratories). Membranes were hybridized to an [a-32P]dCTP-labeled cDNA probe, washed, and visualized by autoradiography. Ethidium
bromide staining was used to ensure equal RNA loading. GAPDH mRNA and 28s RNA were monitored as an internal controls.

**Electrophoretic mobility shift assays**
Nuclear extracts from the H460 cells treated with either Ad-EV or Ad-BP3 were subjected to EMSA analysis of the Egr-1 promoter. Approximately 0.1 pmol of the \( \chi^{32P} \)-dCTP–labeled probe was mixed with 2 to 6 µg of nuclear extract in a total volume of 20 µl and then incubated for 30 min at room temperature. Samples were separated by 5%-polyacrylamide gel electrophoresis and visualized by autoradiography. The consensus DNA sequence of the Egr-1 promoter oligonucleotides used was: 5′-GGCCC TCGCC CCCGC GCCGG G-3′ and 5′-GCCCG GCGCG GGGGC GAGGG-3′.

**Figure S1. Recombinant IGFBP-3 protein inhibits bFGF expression**
bFGF expression levels in H1299, and A549 cells were analyzed 2 days after rBP3 treatment.

**Figure S2. IGFBP-3 suppresses Egr-1 expression**
(A, B) RT-PCR (A) and northern blot (B) analyses of the indicated gene expression in H460 cells that had been treated with rBP3 (A) or infected with Ad-BP3 or Ad-EV (B) for 2 days. GAPDH mRNA and 28s RNA were monitored as an internal controls. (3) Nuclear extracts from the H460 cells treated with either 100pfu/cell of Ad-BP3 or Ad-EV were subjected to EMSA analysis to analyze the effect of Ad-BP3 on Egr-1 protein binding to bFGF promoter.

**Figure S3. IGFBP-3 inhibits phosphorylation of Erk1/2 independent of MEK1/2**
H460 cells were transfected with the indicated concentration of pBP3. The indicated protein expression levels were analyzed by western blot analysis.
Figure S1. Recombinant IGFBP-3 (rBP3) inhibits bFGF expression. bFGF expression levels in H1299 and A549 cells were analyzed 2 days after rBP3 treatment.
Figure S2. IGFBP-3 suppresses Egr-1 expression. (A, B) RT-PCR (A) and northern blot (B) analyses of the indicated gene expression in H460 cells that had been treated with rBP3 (A) or infected with Ad-BP3 or Ad-EV (B) for 2 days. GAPDH mRNA and 28s RNA were monitored as an internal controls. (C) Nuclear extracts from the H460 cells treated with either 100pfu/cell of Ad-BP3 or Ad-EV were subjected to EMSA analysis to analyze the effect of Ad-BP3 on Egr-1 protein binding to bFGF promoter.
Figure S3. IGFBP-3 inhibits phosphorylation of Erk1/2 independent of MEK1/2
H460 cells were transfected with pBP3 and the indicated protein expression levels were analyzed by western blot analysis.