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Angiogenesis Induced by Signal Transducer and Activator of Transcription 5A (STAT5A) Is Dependent on Autocrine Activity of Proliferin*[S]

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Background: FGFs activate STAT5 transcription factor.
Results: In mouse endothelial cells, active STAT5 promotes the production and release of proliferin, which stimulates endothelial cell migration, invasion and tube formation *in vitro*, and angiogenesis *in vivo*.
Conclusion: Proliferin is a secreted pro-angiogenic factor downstream of FGFs and STAT5.
Significance: Proliferin is an autocrine factor likely relevant in physiologic and pathologic angiogenesis.

Multiple secreted factors induce the formation of new blood vessels (angiogenesis). The signal transduction events that orchestrate the numerous cellular activities required for angiogenesis remain incompletely understood. We have shown previously that STAT5 plays a pivotal role in angiogenesis induced by FGF2 and FGF8b. To delineate the signaling pathway downstream of STAT5, we expressed constitutively active (CA) or dominant-negative (DN) mutant STAT5A in mouse brain endothelial cells (EC). We found that the conditioned medium from CA-STAT5A but not from dominant-negative STAT5A overexpressing EC is sufficient to induce EC invasion and tube formation, indicating that STAT5A regulates the secretion of autocrine proangiogenic factors. Conversely, CA-STAT5A-induced conditioned medium had no effect on EC proliferation. Using a comparative genome-wide transcription array screen, we identified the prolactin family member proliferin (PLF1 and PLF4) as a candidate autocrine factor. The CA-STAT5A-dependent transcription and secretion of PLF by EC was confirmed by quantitative RT-PCR and Western blotting, respectively. CA-STAT5A binds to the PLF1 promoter region, suggesting a direct transcriptional regulation. Knockdown of PLF expression by shRNA or by blocking of PLF activity with neutralizing antibodies removed the CA-STAT5A-dependent proangiogenic activity from the conditioned medium of EC. Similarly, the ability of concentrated conditioned medium from CA-STAT5A transfected EC to induce angiogenesis in Matrigel plugs *in vivo* was abolished when PLF was depleted from the medium. These observations demonstrate a FGF/STAT5/PLF signaling cascade in EC and implicate PLF as autocrine regulator of EC invasion and tube formation.

The formation of new blood vessels (angiogenesis) is essential during development and contributes to tumorigenesis and metastasis (1). During the process of angiogenesis, a series of events must be coordinated at a spatial and temporal level. This includes the degradation of the vascular basement membrane, migration and invasion of endothelial cells (ECs)[2] into the perivascular space, endothelial cell proliferation, and vessel maturation (2). Although several potent paracrine angiogenesis inducers (VEGF, FGFs, angiopoietins) and a variety of downstream effector molecules (integrins, matrix metalloproteases) have been identified, intracellular signaling pathways remain incompletely understood. Also, it is unclear how specific components of the angiogenesis cascade (*e.g.* proliferation *versus* migration or invasion) are specifically and differentially regulated.

Fibroblast growth factors bind to and activate FGF receptor tyrosine kinases (FGFR1–4), which signal primarily through the Ras-Raf-MAPK and/or PI3K-Akt pathways (3). Recently, an alternative signaling pathway involving Jak2 and STAT transcription factors has also been implicated in FGF signaling (4, 5). STAT1 is activated in chondrocytes of thanatophoric dysplasia patients by a constitutively active FGFR3 (6). In human umbilical vein EC, FGF2 stimulates STAT3 (5). We have recently reported that STAT5 and to a lesser degree STAT1 but not STAT3 are activated by FGF2 and FGF8b in mouse brain EC (4). In these cells, active STAT5 induces migration, invasion, and tube formation in collagen gels but not proliferation. This apparent separation of proangiogenic signaling pathways prompted us to examine endothelial effector molecules downstream of STAT5.

We report here that STAT5-induced mouse endothelial cell migration, invasion, and tube formation requires the secretion of an autocrine factor and identify this factor as the prolactin family member proliferin (PLF). We show that STAT5 binds to

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[2] The abbreviations used are: EC, endothelial cell; qRT, quantitative real time; PLF, proliferin; BMVEC, brain microvascular endothelial cell; CA, constitutively active; DN, dominant-negative; AP-1, activator protein 1; CM, conditioned medium; PRL, prolactin.

the regulatory region of the PLF1 gene and thus directly participates in the regulation of its expression. We further demonstrate that secreted PLF is required for STAT5-mediated angiogenesis in the Matrigel plug assay *in vivo*. Thus, we describe a novel role for STAT5 in a FGF-STAT5-PLF signaling cascade that facilitates FGF-induced migration, invasion, and tube formation of brain EC.

EXPERIMENTAL PROCEDURES

Cell Culture—Conditionally immortal mouse microvascular ECs from brain (BMVEC), bone, and prostate isolated from H-2Kb-tsA58 mice (gift from Isaiah J. Fidler) (7), which were extensively characterized and shown to retain their EC characteristics, were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, sodium pyruvate, nonessential amino acids, and vitamin solution (Invitrogen) at 33 °C. The cells were cultured at 37 °C for 24 h to abolish the SV40 large T antigen before the experiments.

Antibodies and Reagents—Stock solutions (10.0 mM) of recombinant human FGF2 (Pepro Tech) and recombinant mouse FGF8b (gift from Alan C. Rapraeger, University of Wisconsin) were diluted in DMEM containing 0.2% BSA to their final concentration and added to cultures for different time periods with occasional mixing. All of the monoclonal antibodies and antisera were obtained from commercial sources. Antibodies to STAT5 (C-17) and to proliferin (N-14) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated anti-mouse PLF (BAF1623) was purchased from R & D Systems (Minneapolis, MN). Streptavidin-HRP was obtained from GE Healthcare. An antibody to phospho-STAT5A/B (8-5-2) was obtained from Millipore (Billerica, MA). Antibodies against FLAG (F-3165) and β-actin (AC-74) were from Sigma-Aldrich. Collagen type I and collagen invasion chambers were purchased from BD Biosciences (Sparks, MD).

Adenoviral Plasmids—An adenoviral expression system containing a constitutively active STAT5A mutant (CA-STAT5A) harboring a COOH terminus FLAG tag (pRKmSTAT5-AHSFLAG; generated from pRKmSTAT5AcFLAG using site-directed mutagenesis by substituting His-299 and Ser-711 with arginine and phenylalanine, respectively) and a dominant-negative STAT5A mutant (DN-STAT5A) truncated from amino acids 713 to 793 (pRKmSTAT5A713FLAG) was generated in our laboratory and described previously (4). For adenovirus infection of target cells, BMVEC (5×10^5 cells/well) were seeded into 10-cm Petri dishes 1 day prior to infection. A fixed volume of a viral stock (50 pfu/cell) was used to infect the target cells for 24 h. The infected BMVEC were then starved in serum-free DMEM for 24 h for the subsequent experiments. Conditioned medium was collected for the subsequent experiments as needed.

Lentivirus-mediated PLF-shRNA Expression—PLF expression in BMVEC was silenced with shRNA-containing lentiviral particles commercially obtained from Santa Cruz Biotechnology. Control shRNA (sc-10808) and shRNA targeting PLF (sc-61412-v) consist of a pool of three to five expression constructs each encoding target-specific 19–25-nucleotide (plus hairpin) shRNA designed to knockdown gene expression. The constructs were introduced into the BMVEC by lentiviral transduction, supplementing the medium with 8 μg/ml Polybrene (Sigma-Aldrich). After selection with Puromycin (8 μg/ml; Sigma-Aldrich) for about 2 weeks, the cells were pooled and expanded.

Real Time PCR—Total RNA from treated BMVEC was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed in a reaction volume of 20 μl, containing 2 μl of 10× reverse transcription buffer, 4 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP, 1 μl of 50 μM random primer, 1 μl of 40 units RNaseOUT (Invitrogen). Synthesis of cDNA was carried out for 50 min at 50 °C. PCR amplifications were carried out in a 25-μl reaction volume at a 60 °C annealing temperature. The primer sequences used for the ChIP assays were as follows: F1, CCAACTCCAGTGAAGCA; R1, TCAGTACCTAAGCCGTGTGG; F2, TGAGACTGCTGGTCCTCCTA; R2, TGCTGACAACTGTGGTTGAA; F3, TTGTGAGCCCTGTTTGAGAG; and R3, AGTCTCCTTCACTGCCCATT. The primer sequences used to measure PLF1 gene expression were: F1, TTCAACCATGCTCCTGGATA; and R1, GACCATTCCTCATTGCACAC. The primer sequences used to measure PLF4 gene expression were: F1, CCCTCATGAGCACCATGAA; and R1, TGATCATGCCATATGAAGAAC.

Assessment of Cell Growth—For measuring cell proliferation, 1.5×10^5 BMVEC were seeded into DMEM supplemented with 0.5% or 5.0% FBS in a 24-well plate and cultured at 37 °C in a CO_2 incubator. After a 96-h incubation, the cells were washed with ice-cold saline, trypsinized, stained with Trypan Blue, and counted. For the chromogenic proliferation assay, 3000 cells in a 100-μl volume were seeded into 96-well plates and at the end of treatments, 50 μl of CellTiter 96Aqueous One Solution Reagent (Promega, Madison, WI) were added. The cultures were incubated for 1–4 h at 37 °C, 5% CO_2, and absorbance at 490 nm was recorded using a 96-well plate reader (SpectraMax; Molecular Devices).

Immunoblot—The cells were collected and washed three times with cold PBS and lysed with 150 μl of RIPA buffer supplemented with protease and phosphatase inhibitors on ice for 20 min. Cell lysates were cleared by centrifugation at 13,000 rpm for 20 min at 4 °C. Protein concentration was measured by BCA assay, and the lysates were mixed with 5× sample buffer. After boiling for 5 min, 50 μg of total protein from each sample were separated on a 7.5–12% SDS-PAGE gel. After transfer, the nitrocellulose membranes were blocked with Tris-buffered saline containing 5% nonfat dry milk for 1 h at room temperature and incubated with primary antibodies against the proteins of interest in Tris-buffered saline containing 5% nonfat dry milk plus 0.5% Tween 20, overnight at 4 °C. After washing and incubation with a horseradish peroxidase-conjugated secondary antibody, the proteins were revealed using an enhanced chemiluminescent detection kit (Thermo-Scientific, Rockford, IL).

TCA Precipitation of Samples for SDS-PAGE—The volume of conditioned medium samples was adjusted up 1 ml and precipitated with 100 μl of 100% TCA (28.57 g of TCA in 20 ml of Milli Q water). The tubes were placed on ice for at least 2 h. The samples were centrifuged at 15,000 × *g* for 20 min at 4 °C, and the precipitates were washed twice with cold acetone (−20 °C).

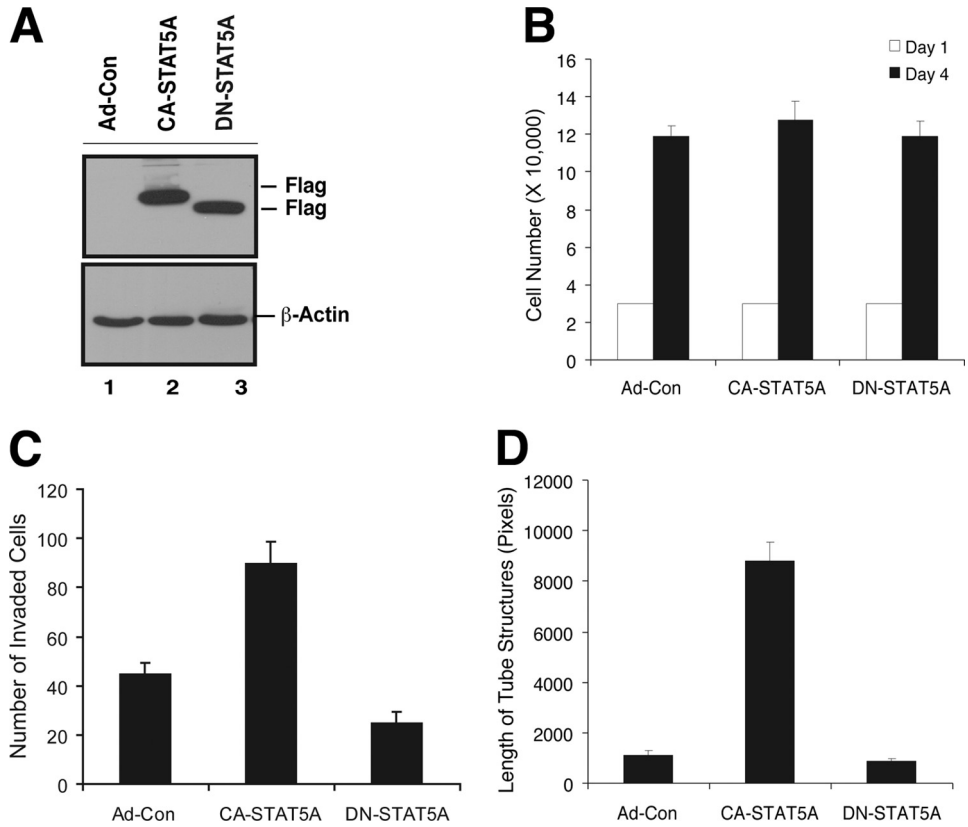

FIGURE 1. **Autocrine activities of STAT5A-overexpressing ECs.** BMVEC were infected with Ad-Con, Ad-CA-STAT5A, or Ad-DN-STAT5A at 50 pfu/cell, and conditioned medium was collected. *A*, confirmation of CA-STAT5A and DN-STAT5A expression. Total protein (25 μg) was loaded in each lane, and exogenous, FLAG-tagged STAT5A was detected by Western blotting. *B*, effect of conditioned medium on EC proliferation. Conditioned medium collected from the cell sources indicated under the columns was added to starved BMVEC, and cell numbers were determined after different time intervals. *C*, effect of conditioned medium on EC invasion. BMVEC invasion was assayed using modified Matrigel invasion chambers. The cells (2×10^5) were added to the upper chamber in serum-free DMEM. Conditioned medium was applied as a chemoattractant to the lower compartment of the chamber. The cells at the lower aspect of the membrane were counted after 3 days. *D*, effect of conditioned medium on EC tube formation in collagen gels. BMVEC were tested for tube formation in collagen gels as detailed under "Experimental Procedures." EC tube formation was measured at 16 h. Photographs were taken with a phase contrast microscope, and relative tube length was measured with ImageJ and expressed as the means ± S.D. for three photographs. Shown is one of three independent experiments.

After briefly air drying, samples were mixed with 1× sample buffer and boiled for 5 min.

ChIP Assay—The assay was performed according to the antibody manufacturer's ChIP assay kit protocol (Millipore). Briefly, 1×10^7 BMVEC (transduced with Ad-Con, Ad-CA-STAT5A, and Ad-DN-STAT5A at 100 pfu/cells) were grown in 100-mm dishes, and cross-linking was accomplished by adding formaldehyde to final concentration of 1% (v/v), incubating at room temperature for 10 min. The samples were washed twice with ice-cold PBS containing protease, and the cells were scraped into conical tubes, pelleted, and resuspended in ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0). After a 10-min incubation on ice, the cells were sonicated to shear DNA to lengths between 200 and 1000 base pairs. After centrifuging for 10 min at 13,000 rpm and 4 °C, the A_{260} of the lysates was measured. Then 60 μl of protein A-agarose beads (Sigma) were added to the sonicated lysates and rotated at 4 °C for 1 h to reduce the nonspecific binding. Anti-STAT5 immunoprecipitation antibody (C17; Santa Cruz Biotechnology) was added to the supernatant and incubated for 2 h at 4 °C on a rotator. As a negative control, the primary antibody was omitted, and the supernatant was incubated with 60 μl of salmon sperm DNA/protein A-agarose. The agarose beads were pelleted by gentle centrifugation (700–1000 rpm at 4 °C, ~1 min) and washed for 3–5 min on a rotating platform with 1 ml of each of the buffers listed in the following order: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl); high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl); LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% SDC, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0). The DNA-transcription factor complex was eluted from the antibody by adding 250 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) to the pelleted protein A-agarose-antibody-STAT5 complex. 5 M NaCl (20 μl) was added to the eluates, and the STAT5-DNA cross-links were reversed by heating at 65 °C for 4 h. Ten μl of 0.5 M EDTA, 20 μl 1 M Tris-HCl, pH 6.5, and 2 μl of 10 mg/ml proteinase K were added to the eluates and incubated for 1 h at 45 °C. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The pellets were washed with 70% ethanol, air-dried, and resuspended in an appropriate buffer for PCR.

RNA Expression Array—BMVEC at 75% confluence were infected with Ad-Con, Ad-CA-STAT5A, or Ad-DN-STAT5 at 100 pfu/cell. The infected cells were cultured for 48 h with complete medium at 33 °C and starved for an additional 24 h

TABLE 1
Genes induced by CA-STAT5A Overexpression
cDNAs were prepared from total RNA, and expression levels were analyzed using the Nimblegen platform as described under "Experimental Procedures." Genes induced 5-fold or higher are listed.

Transcript	Gene symbol	Fold induction	Predicted protein (function)
XM_111219	EG237749	58.6	
BC010818	Sprr2a	24.7	Small proline-rich protein 2A, cornifin family
XM_001000192	LOC668231	22.3	
AK146137	EG226604	19.5	
NM_177744	Apol10a	19.4	Apolipoprotein L 10A
NM_001033335	Serpina3f	19.1	Serine protease inhibitor A3F
AK154426	Gm10522	18.1	
BC049968	Rgs16	17.2	Regulator of G protein signaling 16
BC027400	Aqp3	12.2	Aquaporin 3 (promotes water transport across cell membranes)
NM_001037865	Col28a1	12.1	Collagen type XXVIII α1 (may act as cell-binding protein)
XM_133360	LOC233038	10.7	
AK132394	Slco2b1	10.4	Solute carrier organic anion transporter family, member 2B1
BC063328	Scin	10.1	Scinderin (calcium-dependent actin-severing and -capping protein)
BC056203	Prl2c2 (PLF1)	9.7	Prolactin family 2, subfamily c, member 2; Proliferin-1
BC106153	Socs2	9.3	Suppressor of cytokine signaling 2 (STAT-induced STAT inhibitor 2)
NM_001013824	EG435337	8.0	
NM_053080	Aldh1a3	7.9	Aldehyde dehydrogenase 1 family, member A3
NM_001040611	Peg10	7.7	Paternally expressed 10
BC025512	Rnf183	7.6	Ring finger protein 183
NM_177769	Elmod1	7.2	ELMO/CED-12 domain containing 1
NM_017474	Clca3	7.2	Chloride channel accessory 3 (pseudogene)
BC003783	Cish (Cis)	7.0	Cytokine inducible SH2-containing protein (STAT inhibitor)
NM_181852	Prl2c5	6.8	Prolactin family 2, subfamily c, member 5; Proliferin-4
NM_001037919	EG623898	6.3	
XM_890107	Dub3	6.3	Deubiquitinating enzyme 3
NM_010089	Dub2	5.8	Deubiquitinating enzyme 2
XM_990379	EG667403	5.2	
BC030929	D630039A03	5.1	
NM_010518	Igfbp5	5.0	Insulin-like growth factor-binding protein 5
XM_892460	EG546539	5.0	
XM_994582	EG666862	5.0	

after two washes with PBS. Total RNA from 5×10^5 cells for each sample was extracted using the RNeasy microRNA kit (Qiagen). Each sample was analyzed on a NanoDrop 1000 spectrophotometer and Agilent RNA 6000 NanoChip to verify RNA quantity and quality, respectively, and RNA from three independent experiments was pooled. Ten μg of total RNA per sample were used for cDNA synthesis using oligo$(dT)_{15}$ as primer (Promega). After adding EtOH to precipitate the cDNA, the samples were stored overnight at −20 °C. The cDNA samples were purified and quantified with a NanoDrop 1000 spectrophotometer, and the size distribution determined on an Agilent RNA 6000 NanoChip. The cDNA samples were then sent to Roche NimbleGen (Madison, WI) for mouse gene expression array analysis on a fee-for-service basis.

We jointly analyzed RMA ("robust multichip analysis") (8) normalized intensity values from the three experiments (CON = empty virus, CA = CA-STAT5A, DN = DN-STAT5A), using an empirical Bayes method for gene expression data (9). This method grouped genes into five different patterns (P1: CON = CA = DN; P2: CON = CA! = DN; P3: CON! = CA = DN; P4: CA! = CON = DN; P5: CON! = CA! = DN) based on their posterior probabilities of belonging to these prespecified groups. We further summarized the genes that are differentially expressed between CON and CA (and differentially expressed between CA and DN) by combining data sets from P4 and P5. Genes expressed at low levels in CA (arbitrary signal level of 200 or less) were filtered out. Fold change (CA/CON) was calculated, and an arbitrary value of 5 was used as the cut-off.

Collagen Gel EC Tube Formation Assay—Collagen gels were prepared according to a previous report (4). Briefly, a 12-well tissue culture plate was prechilled at −20 °C and coated with rat tail collagen I (1.3 μg/ml; 500 μl/well; BD Biosciences). The plate was incubated at 37 °C for 1 h to allow the collagen to solidify. Brain EC (15,000 cells/well) were seeded on the surface of the collagen gel in starvation medium. After 12–24 h, images of the tube structures were captured under phase contrast microscopy using a SPOT RT Slider digital camera (Diagnostic Instruments, Sterling Heights, MI) and analyzed using ImageJ software. Tube length was assessed by drawing a line along each tubule and measuring the length of the line in pixels. Tube length was measured for each sample in five nonoverlapping fields under 200× original magnification.

Monolayer Wound Healing Assay—BMVEC were seeded onto 6-well plates at 5×10^5 cells/well and grown to confluence prior to a 24-h starvation period in serum-free DMEM. A single scratch wound was introduced in the monolayer using a micropipette tip, and the medium was replaced with conditioned medium from differently treated BMVEC. Wound closure was monitored for 48 h.

EC Invasion Assay—BMVEC invasion was assayed using modified invasion chambers with polycarbonate PVP-free Nucleopore filters (8 μm pore size), coated with 25 μg/filter Matrigel (BD Bioscience). Starved BMVEC (2×10^5 cells/well) without treatment or infected with Ad-Con, Ad-CA-STAT5A, or Ad-DN-STAT5A and suspended in serum-free DMEM were added to the upper chamber. Medium containing 0.2% FBS or conditioned media from STAT5A overexpressing BMVEC was placed in the lower chamber as a chemoattractant. At the end of a 48-h incubation period, the cells on the upper surface of the filter were removed with a cotton swab, and cells on the lower surface of the filter were stained with Hoechst 33342 (1 μg/ml).

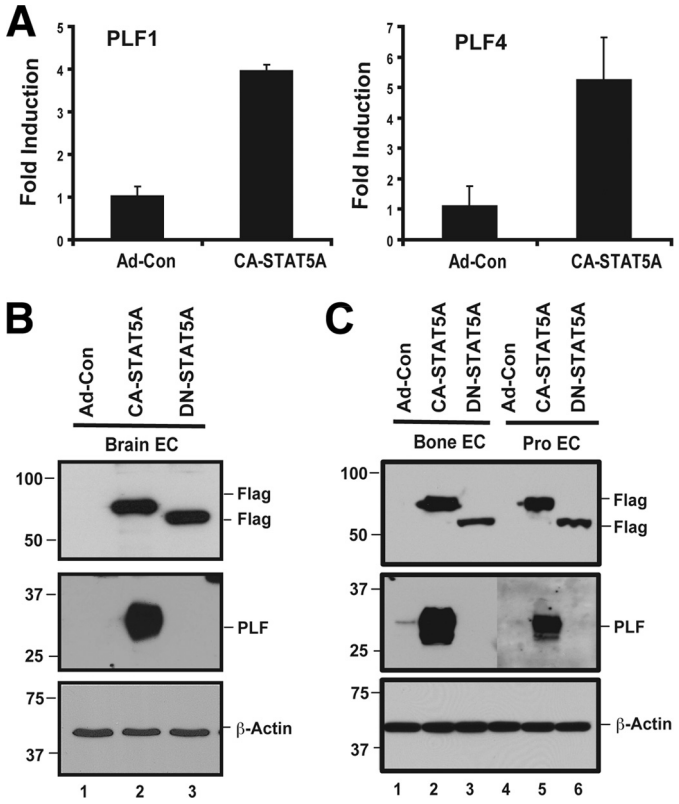

FIGURE 2. **STAT5A activates PLF expression.** *A*, induction of PLF1 and PLF4 mRNA by active STAT5A. PLF1 and PLF4 mRNA was analyzed by qRT-PCR using specific primers after adenoviral transduction with CA-STAT5A. *B*, CA-STAT5A-induced PLF protein expression in conditioned medium detected by Western blot. Conditioned media from Ad-Con, Ad-CA-STAT5A, and Ad-DN-STAT5A-transduced BMVEC were TCA-precipitated and resolubilized in sample buffer as described under "Experimental Procedures." Equal amounts of protein were fractionated on SDS-PAGE gels, and the membranes were probed with antibodies to mouse PLF. The antibody used in this and other Western blots does not differentiate between PLF1 and PLF4. Cell lysates were also analyzed for exogenous expression of FLAG-tagged STAT5, and β-actin served as loading control. *C*, STAT5A activates PLF expression in bone and prostate endothelial cells. CA-STAT5A-induced PLF protein expression in conditioned medium detected by Western blot. Conditioned media from Ad-Con, Ad-CA-STAT5A, and Ad-DN-STAT5A-transduced bone ECs and prostate ECs were TCA-precipitated and resolubilized in sample buffer as described under "Experimental Procedures." Equal amounts of protein were fractionated on SDS-PAGE gels, and the membranes were probed with antibodies to mouse PLF. The cell lysates were also analyzed for exogenous expression of FLAG-tagged STAT5.

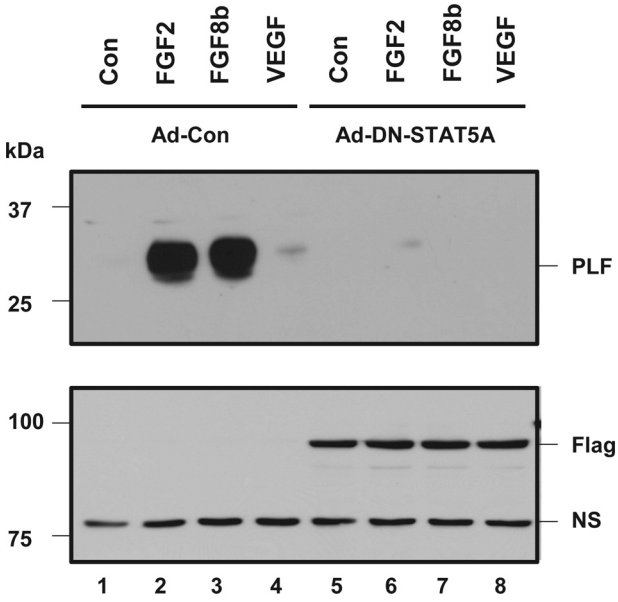

FIGURE 3. **FGF2, FGF8b, and VEGF stimulate PLF expression, whereas DN-STAT5A blocks this induction.** BMVEC at 90% confluence were transduced with Ad-Con or Ad-DN-STAT5A at 50 pfu/cell. After adenoviral transduction, the cells were incubated for 48 h and then starved for an additional 24 h. The medium was then replaced with fresh serum-free DMEM alone (control) or supplemented with FGF2 (10 nM), FGF8b (10 nM), and VEGF (20 ng/ml) for 6 h. Conditioned medium was precipitated using the TCA method as described under "Experimental Procedures," and equal amounts of precipitated protein were analyzed for PLF expression by Western blot as indicated. DN-STAT5A was detected through its FLAG tag. *NS* indicates a nonspecific band. FGF2 and FGF8b treatment strongly induced PLF expression, whereas VEGF was less potent. DN-STAT5A abolished growth factor-induced PLF expression.

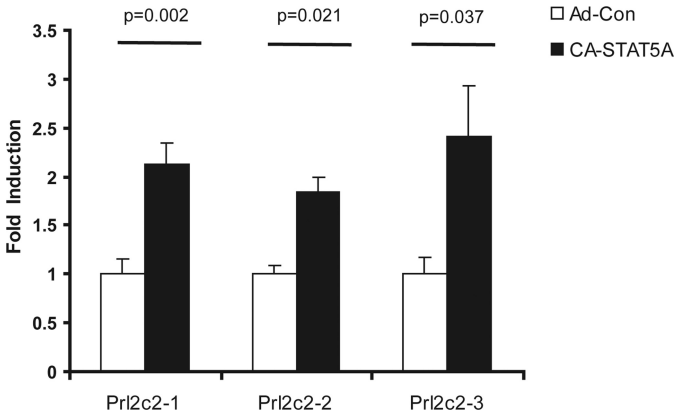

FIGURE 4. **Active STAT5A binds to the PLF1 promoter.** Chromatin immunoprecipitation with a STAT5-specific antibody was performed as described under "Experimental Procedures." DNA fragments in the precipitates were analyzed by qRT-PCR using three primer pairs designed to amplify the PLF1 (*prl2c2*) promoter region. CA-STAT5A overexpression significantly increases STAT5 binding to the PLF1 promoter region ($p = 0.002$ for primer pair 1; $p = 0.021$ for primer pair 2; and $p = 0.036$ for primer pair 3). The results shown are representative for one of four independent experiments.

Cells on the lower surface were counted, and each assay was performed in triplicate.

In Vivo Matrigel Plug Assay—Conditioned media (5 ml each) from STAT5A overexpressing MBVEC were incubated with goat anti-mouse PLF antibody (60 μg; Santa Cruz Biotechnology) or an equivalent amount of goat serum at 4 °C for 2 h and then depleted with 300 μl of protein A beads for 3 h at 4 °C. The supernatant volumes were reduced to 2 ml by vacuum concentration for about 1 h at 4 °C. The resultant preparations were then analyzed for PLF content by TCA precipitation followed by Western blot. Aliquots of PLF-depleted conditioned media were then mixed with Matrigel at a 1:1 ratio yielding a final Matrigel concentration of 6.5 mg/ml. Then 0.6 ml of the media-Matrigel mixture were injected subcutaneously into the bilateral flanks of athymic nude mice (6–8 weeks). Each group contained three mice (six plugs in total). The same volumes and concentrations of Matrigel containing 20 ng/ml FGF2 and 1 μg/ml heparin, with or without goat anti-PLF antibody (final concentration, 10 μg/ml; goat serum served as control), were injected into another group of mice as controls. Matrigel plugs were harvested after 8 days, photos were taken, and the plugs were fixed in 10% formalin, processed, and embedded in paraffin to assess EC invasion.

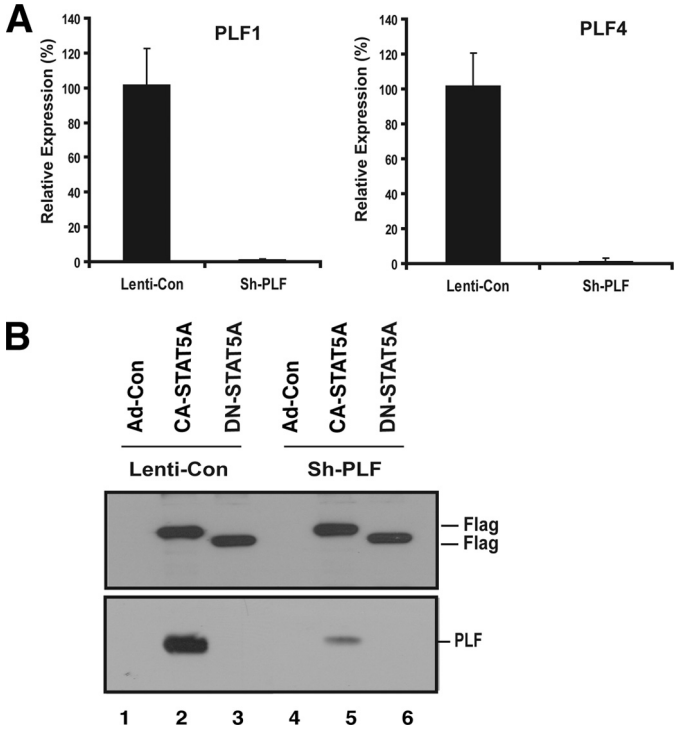

FIGURE 5. **STAT5-activated PLF expression is suppressed by PLF-shRNA.** To disrupt the expression of PLF, a pool of shRNA constructs targeting PLF were introduced into BMVEC via lentivirus. *A*, PLF shRNAs diminish PLF1and PLF4 mRNA levels. PLF1 and PLF4 mRNA was detected by qRT PCR in EC treated with shRNA lentivirus particles or control. *B*, PLF shRNAs reduce CA-STAT5A-induced PLF protein expression. shRNA-expressing cells were infected with Ad-Con, Ad-CA-STAT5A, or Ad-DN-STAT5A at 50 pfu/cells. The cultures were washed twice with PBS, and serum-free medium was replaced 48 h after adenoviral transduction. Conditioned medium was collected after an additional 24-h incubation period, and protein in the medium was precipitated with the TCA method. The resolubilized material was analyzed with a 12% acrylamide gel, and the membranes were probed for PLF. The membranes were also probed with an anti-FLAG antibody to detect exogenous mutant STAT5A.

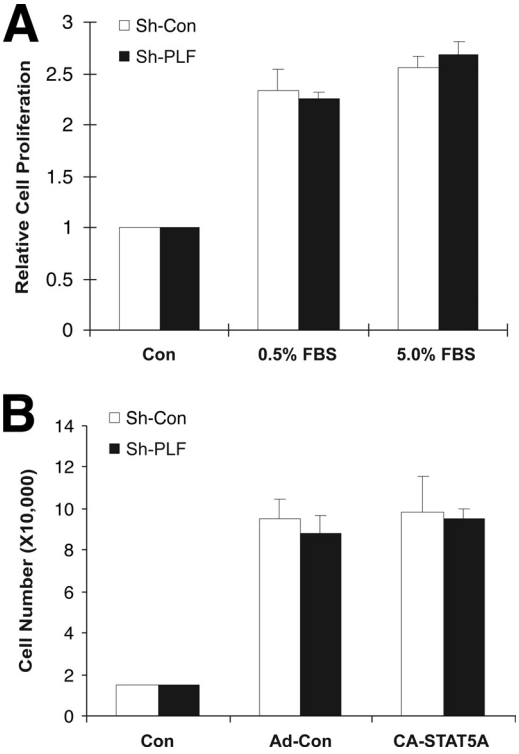

FIGURE 6. **PLF is not required for BMVEC mitogenesis.** *A*, BMVEC transduced with PLF shRNA or control were plated in media containing different serum concentrations, and cell growth was assessed with a chromogenic proliferation assay. The data represent the means ± S.D. of two experiments. Control (*Con*) represents the number of cells on day 0. *B*, BMVEC expressing PLF shRNA were transduced with adenovirus carrying CA-STAT5A or empty control adenovirus. Ninety-six hours later, the cells were harvested, and viable cells were counted by Trypan blue exclusion or using the One-Solution cell proliferation assay kit (Promega). The data represent the means ± S.D. of two experiments. Control (*Con*) represents the number of cells on day 0.

RESULTS

STAT5 Activation Induces Secretion of Proangiogenic Factor by EC—We have recently shown that STAT5 orchestrates FGF-induced endothelial cell migration, invasion, and tube formation (4). To investigate the possibility that this activity involves a secreted factor, we expressed CA-STAT5 or DN-STAT5 in BMVEC by adenoviral transduction (Fig. 1*A*), collected conditioned medium, and added it to native BMVEC cultures. Consistent with our previously reported observations (4), conditioned media from CA-STAT5-transduced cells did not affect endothelial cell proliferation compared with conditioned media from cells treated with empty virus or DN-STAT5-transduced cells (Fig. 1*B*). However, conditioned media from CA-STAT5-transduced cells stimulated BMVEC invasion (Fig. 1*C*) and tube formation (Fig. 1*D*), two processes essential for angiogenesis. These observations indicate that STAT5 activation in EC leads to the secretion of a single or multiple factors that specifically promote invasion and tube formation but not mitogenesis via an autocrine loop.

STAT5 Activation Induces Secretion of Proliferin (PLF)—To identify the factor (or factors) secreted in response to STAT5 activation, we compared gene expression signatures between BMVEC transduced with CA-STAT5A, empty virus or DN-STAT5A using the NimbleGen platform and an empirical Bayes analysis method. After eliminating genes expressed at low levels (see "Experimental Procedures" for details), we identified 31 transcripts (47 probe sets) encoding 20 unique genes that were overexpressed in CA-STAT5 at least 5-fold (Table 1). Two of the genes (Cish and Socs2) are known STAT5 targets; others, involved in intracellular signaling (SPrr2a, Rgs16, and ElmoD1) or the ubiquitination pathway (Dub2 and Dub3), have not previously been linked to STAT5 signaling. Interestingly, in the context of these studies, at least six of the transcripts encode secreted proteins (Apo10a, Serpina3f, Col28a1, Prl2c2, Prl2c5, and Igfbp5). qRT-PCR confirmed the induction of the transcription of Prl2c2 (PLF1) and Prl2c5 (PLF4) by CA-STAT5 (Fig. 2*A*). Importantly, Western blotting with an antibody that detects mouse PLF (PLF1 and PLF4) demonstrated the secretion of PLF into the conditioned medium of BMVEC transduced with CA-STAT5 (Fig. 2*B*). The induction of PLF expression was not limited to BMVEC cells but was also observed in ECs from bone and prostate (Fig. 2*C*). Unless otherwise specified, we refer subsequently to PLF without differentiating between PLF1 and PLF4, which share a high degree of homology.

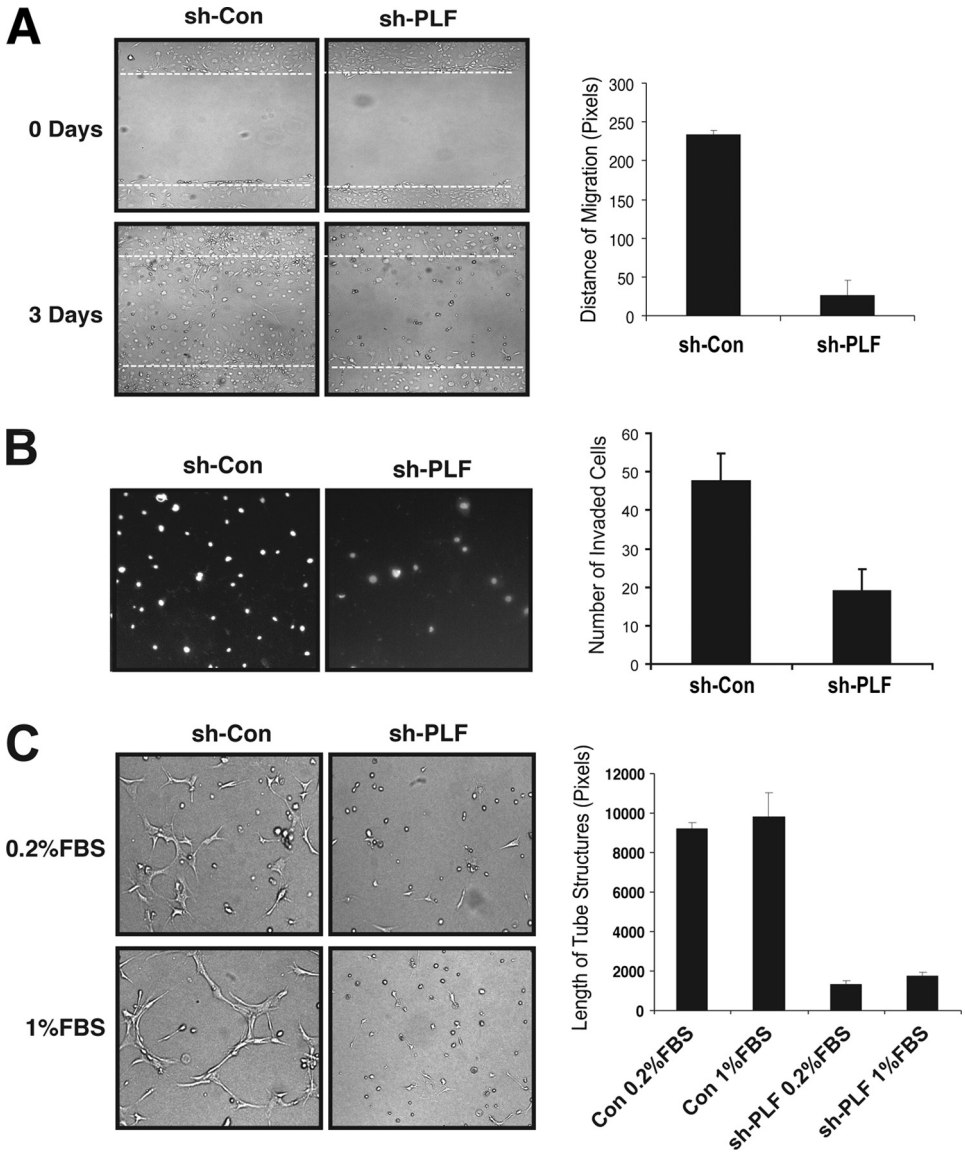

FIGURE 7. **PLF is necessary for serum-induced EC migration and tube formation.** *A*, dependence of BMVEC migration on PLF. BMVEC stably expressing PLF shRNA (*sh-PLF*) or a control construct (*sh-Con*) were cultured in 6-well plates. Cell monolayers at ~95% confluence were wounded with a pipette tip, after a 24-h starvation period and 24 h post-transduction with adenovirus (*upper panels*). The medium was then replaced with DMEM plus 0.2% FBS, and the cells were incubated for three additional days (*lower panels*). Relative migration distance during the gap closure was measured with ImageJ (*bar graph*). PLF shRNA significantly diminished serum-induced migration ($p < 0.01$). *B*, role of PLF in BMVEC invasion. The invasion of BMVEC expressing PLF shRNA or the shRNA control construct was measured in modified Matrigel invasion chambers. Starved BMVEC were added to the upper chamber in serum-free medium. Serum-containing (0.2% FBS) medium was applied as a chemoattractant in the lower compartment of the chamber. The cells located at the lower aspect of the membrane were counted after 48 h. *C*, PLF expression is required for EC tube formation in collagen gels. BMVECs expressing PLF shRNA or shRNA control construct were tested for tube formation in collagen gels in the presence of FBS (0.2% or 1%) as detailed under "Experimental Procedures." EC tube formation was measured at 12 h. Photographs were taken with a phase contrast microscope (*panels*), and relative tube length was measured with ImageJ and expressed as the means ± S.D. after analyzing three photographs (*bar graph*). Silencing of PLF expression resulted in decreased tube formation ($p < 0.01$). Shown is one of three independent experiments.

FGF2, FGF8b, and VEGF Induce PLF Expression in STAT5-dependent Manner—We have previously observed the activation of STAT5 after exposing EC to FGF2 or FGF8b (4). Therefore, we sought to determine whether these growth factors and VEGF, another powerful angiogenesis stimulator, induce PLF secretion. Indeed, abundant PLF is detected in the conditioned medium of BMVEC treated with FGF2 or FGF8b (Fig. 3). Only minimal PLF secretion is seen in response to VEGF, which may be due to a loss of VEGF signaling pathway components in the immortalized endothelial cell line used in these experiments. Importantly, transduction with DN-STAT5 abolishes the growth factor-induced secretion of PLF. Together, these observations are consistent with the novel finding that STAT5 activation is both necessary and sufficient for FGF-induced PLF secretion.

STAT5 Binds to PLF1 Promoter—It has been reported that inducible PLF gene transcription in response to serum growth factors involves activator protein 1 (AP-1) (12–14); however, the regulation of PLF expression remains incompletely understood. To test whether STAT5 participates directly in the transcriptional regulation of PLF, we examined STAT5 binding to the PLF1 promoter by ChIP. Transduction of BMVEC with

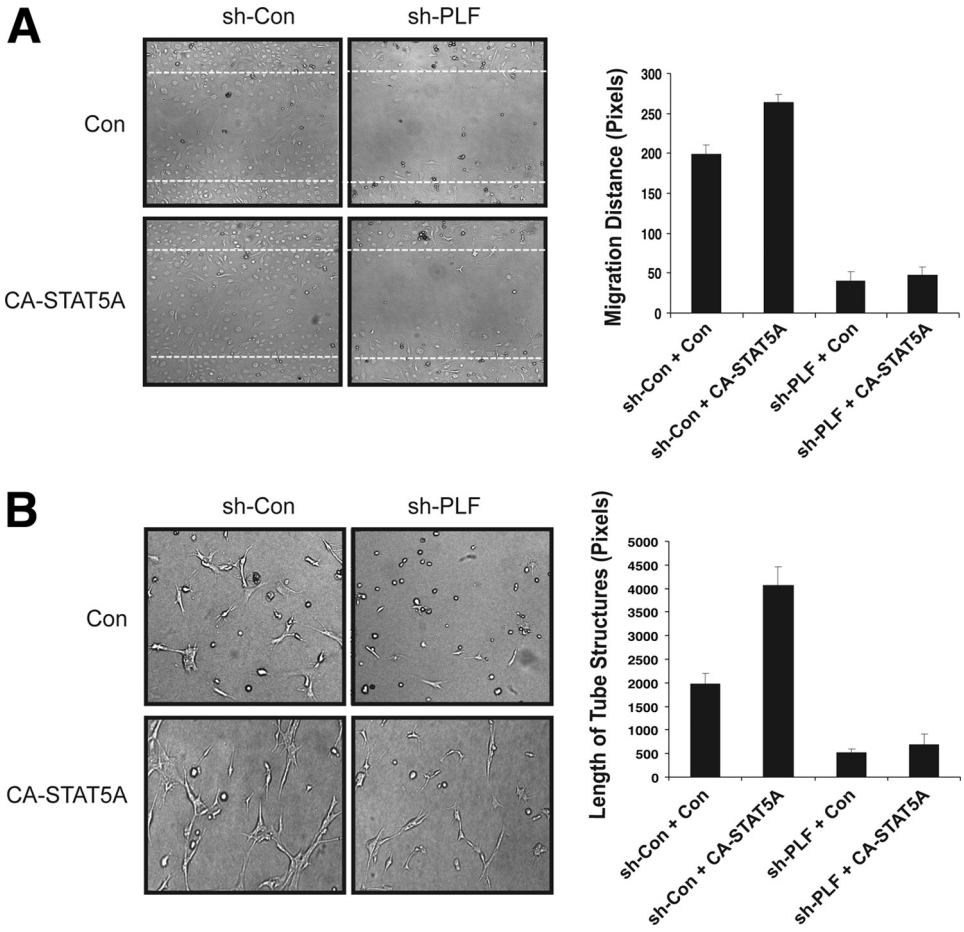

FIGURE 8. **PLF is required for STAT5A-induced EC migration and tube formation.** *A*, dependence of EC migration on PLF. BMVEC that stably express PLF shRNA (*sh-PLF*) or shRNA control construct (*sh-Con*) were infected with empty adenovirus (*Con*) or adenovirus delivering CA-STAT5A (100 pfu/cell). The cell monolayer wounding assay was performed as described for Fig. 7 except that the medium was supplemented with BSA (0.5% m/v). CA-STAT5A increased cell migration compared with Ad-Con ($p < 0.01$). PLF shRNA abolished CA-STAT5A-induced migration ($p < 0.01$). *B*, PLF is required for STAT5A-induced EC tube formation. BMVEC stably expressing PLF shRNA or shRNA control construct were transduced with Ad-CA-STAT5A or empty virus and were tested for tube formation in collagen gels as detailed under "Experimental Procedures." These experiments were performed in the presence of BSA (0.5% m/v). EC tube formation was measured at 12 h. Photographs were taken with a phase contrast microscope, and relative tube length was measured with ImageJ. The results are expressed as the means ± S.D. for three photographs (*bar graph*). CA-STAT5A-stimulated tube formation was diminished by PLF shRNA, indicating that PLF is required for this activity ($p < 0.01$). Shown is one of three independent experiments.

CA-STAT5 led to a significant increase in STAT5 binding to the PLF1 promoter region, as detected by qRT-PCR using three different primer pairs (Fig. 4). These observations suggest that STAT5 binds to the PLF1 gene in an activation-dependent manner.

PLF Activity Is Required for STAT5-induced Endothelial Cell Migration, Invasion, and Tube Formation—To examine a potential role for PLF in endothelial cell migration, invasion, and tube formation, we decided to disrupt expression of this protein by RNAi. shRNA constructs targeting PLF were stably expressed in BMVEC by lentiviral transduction followed by selection in Puromycin. qRT-PCR demonstrated a 95–99% reduction of baseline PLF1 and PLF4 expression (Fig. 5*A*). Next, we examined whether the shRNA construct can suppress PLF induction by active STAT5. shRNA expressing clones and controls were transduced with CA-STAT5A or DN-STAT5A or control virus. CA-STAT5A-induced PLF protein secretion was dramatically suppressed by PLF shRNA (Fig. 5*B*, *lower panel*). The cells expressing mutant STAT5A and/or PLF shRNA were also lysed and processed for Western blotting. Ectopically expressed STAT5A was detected with an antibody to the FLAG epitope (Fig. 5*B*, *upper panel*).

To address the biological function of secreted PLF in endothelial cell biology, we first examined its role in mitogenesis. As expected, silencing of PLF had no effect on BMVEC proliferation either in the presence of serum or after the cells had been transduced with CA-STAT5A (Fig. 6). This observation is congruent with our previous observation that the STAT5 signaling cascade does not regulate EC proliferation (Fig. 1*B*) (4). To investigate further the role of STAT5-dependent PLF secretion in the regulation of angiogenic events, we analyzed endothelial cell migration, invasion, and tube formation as *in vitro* angiogenesis surrogates. In the monolayer scratch wound migration assay, gap closure was dramatically decreased when PLF expression was silenced (Fig. 7*A*), indicating that efficient endothelial cell migration requires this secreted factor. Similarly, PLF expression silencing inhibited invasion through Matrigel-coated membranes in the absence of other chemo-attractants (Fig. 7*B*).

Endothelial cell tube formation in collagen gels is considered one of the most relevant *in vitro* angiogenesis assays (15).

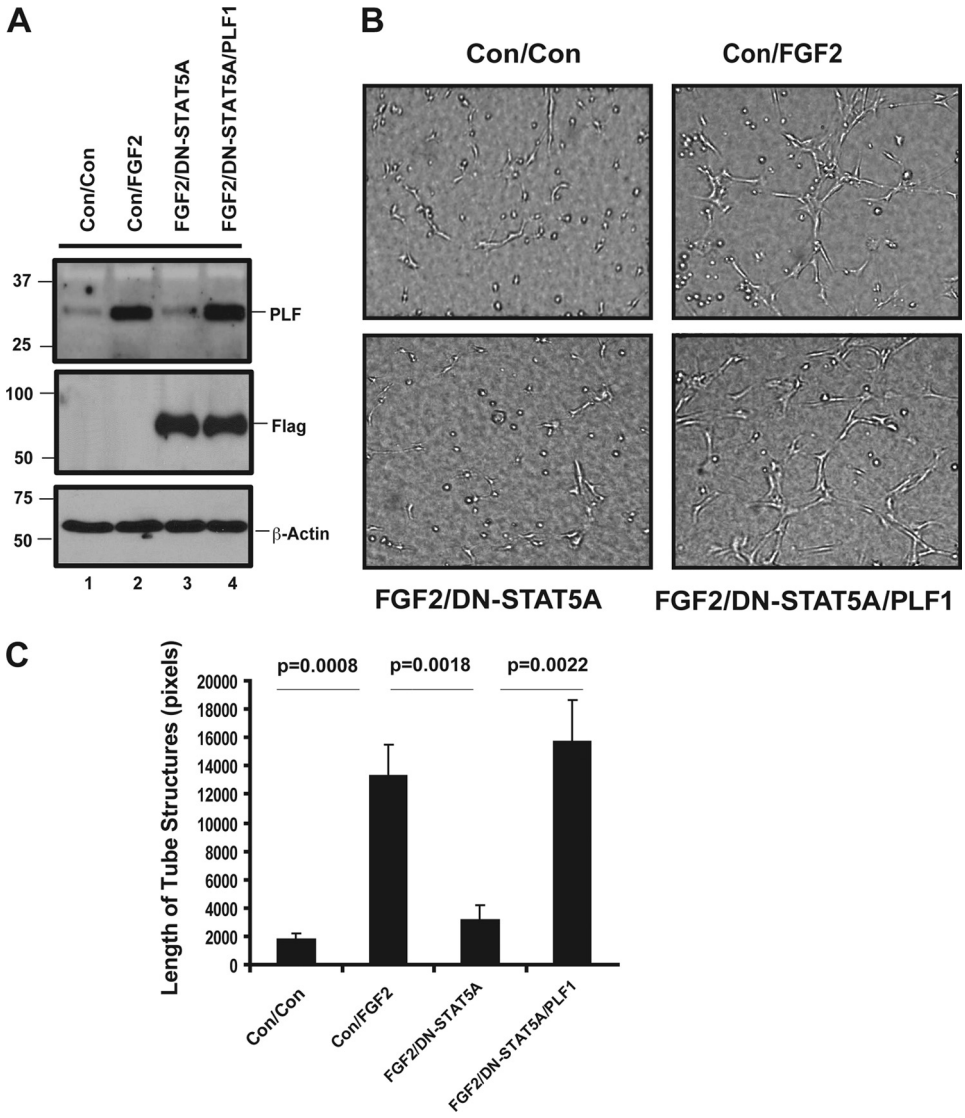

FIGURE 9. **PLF1 expression is sufficient to restore EC tube formation suppressed by DN-STAT5A.** BMVECs were transfected with pcDNA4-PLF1 or empty vector control. The transfected cells were selected with Zeocin at 500 μg/ml to establish stable clones. The pooled clones were expanded and transduced with DN-STAT5A adenovirus (50 pfu/cell). Thirty-six hours post-infection, the cells were starved for 12 h and stimulated with FGF2 at 10 nM for an additional 12 h. Then FGF2 was removed, and the cells were incubated with serum-free DMEM for another 16 h to generate conditioned media. The conditioned media were tested for expression of PLF1 and employed for the tube formation assay in triplicate. The experiments were independently repeated twice using the same conditions as described for Fig. 1. *A*, Western blot to detect proliferin and FLAG-tagged DN-STAT5A. *B*, phase contrast images of BMVEC in collagen gels. *C*, quantitation of tube length for the experiment shown in *B*. Forced expression of PLF1 rescues DN-STAT5A-mediated suppression of FGF-2-induced tube formation.

Silencing of PLF expression with shRNA suppressed serum-induced tube formation (Fig. 7*C*), suggesting that this secreted factor is required for capillary morphogenesis. PLF was also sufficient for EC tube formation, because forced expression of PLF1 in the absence of serum led to a more than 10-fold increase in tube length (supplemental Fig. S1). Further, we examined whether PLF expression silencing can block STAT5-induced angiogenic activities *in vitro*. Cell migration (Fig. 8*A*) and tube formation (Fig. 8*B*) of BMVEC transduced with CA-STAT5 were significantly inhibited by the disruption of PLF expression, implicating PLF as a proangiogenic mediator downstream of STAT5A.

To characterize more definitively the FGF2-STAT5-PLF1 signaling cascade, we stimulated BMVEC with FGF2 and transduced/transfected with DN-STAT5A and PLF1 (Fig. 9*A*). As expected, FGF2 induced tube formation, which was abolished by DN-STAT5A. Forced expression of PLF1 restored tube formation in cells treated with FGF2 and transduced with DN-STAT5A (Fig. 9, *B* and *C*). Together, these data indicate that PLF1 is necessary and sufficient for FGF2/STAT5A-induced EC tube formation.

Neutralization of PLF Abolishes Ability of Medium Conditioned by CA-STAT5A Expressing EC to Induce Endothelial Cell Invasion and Tube Formation—To confirm that the effect of active STAT5 on EC behavior is due to secreted PLF, we added anti-PLF neutralizing antibody to medium conditioned by CA-STAT5-expressing EC and added this medium to wild-type BMVEC. Removal of PLF activity by antibody neutralization reduced CA-STAT-induced endothelial cell invasion ($p < 0.01$; Fig. 10*A*) and tube formation ($p < 0.01$; Fig. 10*B*) to

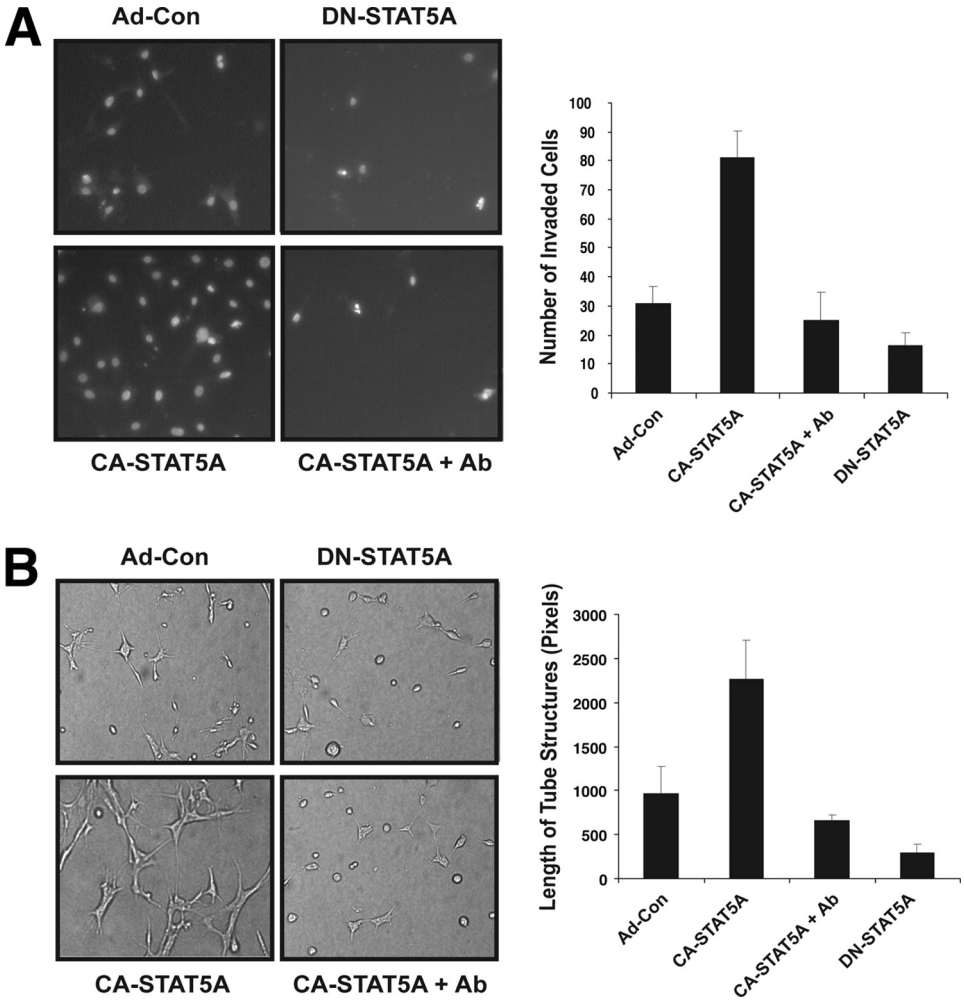

FIGURE 10. **PLF is responsible for the paracrine activity secreted by CA-STAT5A-expressing EC.** *A*, neutralizing antibody to PLF decreases endothelial cell invasion stimulated by conditioned medium from CA-STAT5A-expressing EC. BMVEC invasion was assayed using modified Matrigel invasion chambers. Starved BMVEC (2×10^5) were added to the upper chamber in serum-free medium. Conditioned medium from BMVEC transduced with control virus (*Ad-Con*) or virus delivering mutant STAT5A (CA-STAT5A or DN-STAT5A) was applied as a chemoattractant to the lower compartment of the invasion chamber. Neutralizing antibody to mouse PLF or goat serum control were added to the conditioned medium at a final concentration of 2 μg/ml. Cells at the lower aspect of the membrane were counted after 48 h. The addition of anti-PLF antibody significantly inhibited invasion ($p < 0.01$). *B*, neutralizing antibody to PLF abolishes EC tube formation stimulated by conditioned medium from CA-STAT5A-expressing BMVEC. Assays were performed as described for Fig. 1*D*. Neutralizing antibody directed against mouse PLF or goat serum control was added to conditioned medium produced by CA-STAT5A expressing BMVEC at a final concentration of 2 μg/ml. The antibody-treated conditioned medium was added to starved BMVEC, and tube formation was measured after 12 h. Photographs were taken with a phase contrast microscope, and relative tube length was measured with ImageJ and expressed as the means ± S.D. analyzing three photographs (*bar graph*). The addition of anti-PLF antibody significantly inhibited tube formation ($p < 0.01$).

control levels, suggesting that the majority of the STAT5 effect was mediated by secreted PLF acting on EC in an autocrine fashion.

PLF Stimulates Angiogenesis in Vivo—To further examine the significance of the STAT5-PLF signaling cascade in angiogenesis, we studied PLF activity *in vivo* using a subcutaneous Matrigel plug assay (16). Concentrated conditioned media (CM) from STAT5A-overexpressing BMVEC and from anti-PLF antibody-depleted conditioned medium (Fig. 11*A*) were mixed with Matrigel and inoculated subcutaneously into the flanks of athymic mice. Upon gross inspection, after 8 days, the Matrigel plugs from the PLF antibody-depleted CM group look transparent compared with the red and opaque plugs in the control-treated CM group (Fig. 11*B*). Immunohistochemical labeling with an antibody to the endothelial marker CD31 reveals that PLF depletion significantly reduces endothelial cell area fraction in the Matrigel plugs (Fig. 11, *C* and *D*; $p = 0.012$). As expected, supplementation of the Matrigel with FGF2 produces a robust angiogenic response. The addition of anti-PLF antibody to FGF2-containing Matrigels did not significantly decrease the vessel area fraction (Fig. 11*D*; $p = 0.065$), suggesting that secreted PLF is not the sole downstream effector in FGF2-induced angiogenesis.

DISCUSSION

We have previously shown that both FGF2 and FGF8b, two potent inducers of angiogenesis, activate STAT transcription factors, in particular STAT5 (4). Importantly, FGF-induced STAT5 activation is necessary and sufficient for brain EC migration, invasion and tube formation, all of which are critical components of the angiogenic cascade. In this study we characterize this signaling pathway further and identify a novel role

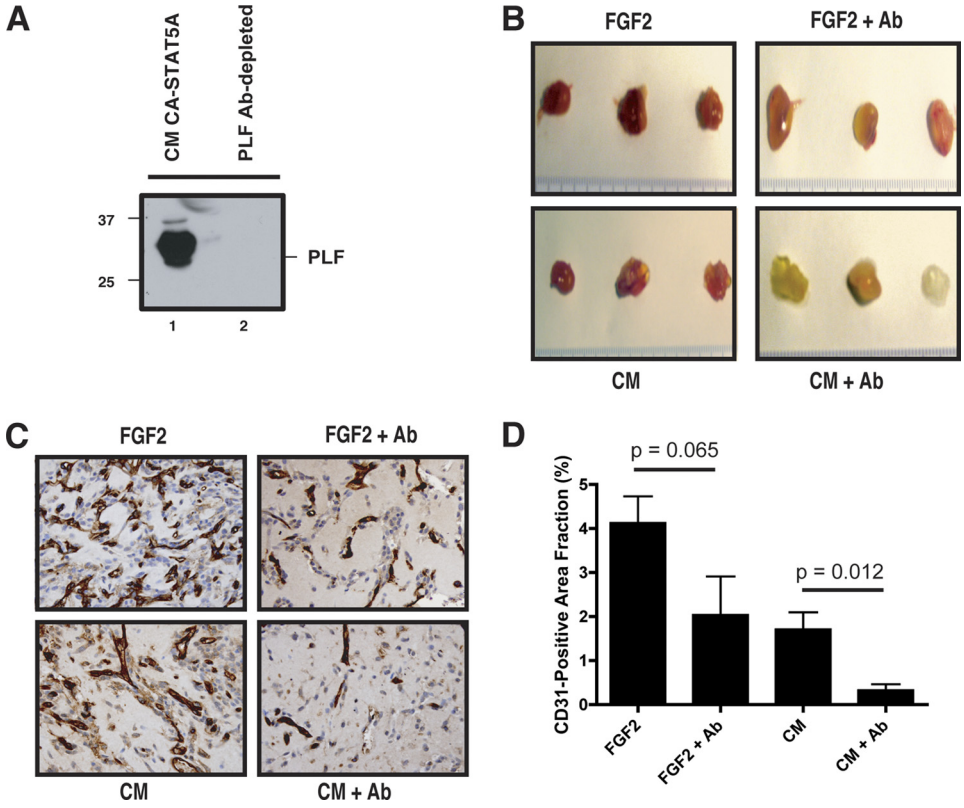

FIGURE 11. **PLF is required for the *in vivo* proangiogenic activity of conditioned medium produced by CA-STAT5A-expressing BMVEC.** Conditioned medium from STAT5A overexpressing BMVEC was treated with goat anti-PLF1 (*CM + Ab*) or goat serum control (*CM*) followed by exposure to protein A-agarose beads to remove PLF1. The conditioned medium was then mixed with Matrigel (see "Experimental Procedures" for details) and injected bilaterally into the flanks of athymic mice (three mice, six plugs/group). As a positive control, Matrigel containing FGF2 (20 ng/ml) and heparin (1 μg/ml), with (FGF2 + Ab) or without (FGF2) anti-PLF antibody were injected into another group of mice. Matrigel plugs were harvested after 8 days, macroscopic photos were taken, and the plugs were fixed in formalin. *A*, Western blot to detect PLF in conditioned medium with and without antibody depletion. *B*, macroscopic images of Matrigel plugs. *C*, sections of Matrigel pugs labeled with an antibody against the endothelial cell marker CD31. PLF depletion of conditioned medium results in decreased plug vascularization. *D*, quantitation of vessel area fraction in the Matrigel plugs. The CD31-positive area was measured using ImageJ software and expressed as a fraction relative to total image area (means ± S.D.). The nonparametric Mann-Whitney test was used to compare antibody treatment groups with their respective control group.

for STAT5 in the induction of the proangiogenic PLF. Thus, we define an autocrine role for PLF in FGF-induced migration, invasion, and tube formation of EC.

PLFs belong to the prolactin/growth hormone/placental lactogen family of polypeptide hormones, which are primarily produced in the pituitary gland and the placenta in most species. To date, four PLF family members have been cloned (plf1, plf2, mrp3/plf3, and mrp4/plf4) (20).

PLF was originally described as a protein secreted by embryonic fibroblasts in response to serum growth factors (17) and has been identified in a mouse placenta expression library (18). The MRP/PLF1–3 proteins are ~98% homologous in their amino acid sequence and have a very similar structure (20). Based on its location in the placenta and functional observations, PLF was thought to participate in fetal-maternal communication and ultimately fetal well-being. PLF is produced by deeply invasive trophoblast giant cells during mid-pregnancy (19), and Jackson *et al.* (11) demonstrated that the protein promotes angiogenesis *in vitro* and *in vivo*. Interestingly, proliferin-related protein suppresses angiogenesis, possibly by antagonizing PLF activity (11). PLF expression is also detected in other organ sites and cell types (20–22). A role for PLF in tumor angiogenesis was first proposed by Toft *et al.* (10), who found that fibrosarcoma cells secrete increasing amounts of proangiogenic PLF while progressing to a more aggressive phenotype. Tumor cell-derived PLF could add to PLF released by EC to promote tumor angiogenesis *in vivo*. Notably, sarcomas, gliomas, and many other malignancies secrete FGF2 (23, 24), which may stimulate angiogenesis directly and/or by promoting endothelial PLF production, as shown here.

PLFs belong to a large family of paralogous murine genes that also includes prolactin (PRL), growth hormone, and placental lactogen (25). Efforts to identify a human PLF homologue have failed to date, and PRL appears to carry out many PLF functions. Different isoforms and proteolytic fragments of PRL have been found to regulate angiogenesis both as stimulators and inhibitors of vessel formation, thus mirroring the antagonistic activities of PLF and proliferin-related protein (11, 26). Castilla recently found that PRL, secreted into ovarian follicular fluid, stimulates endothelial cell proliferation. Although this finding is consistent with roles for PRL family members as stimulators of angiogenesis, it conflicts with our observation that PLF stimulates EC migration and tube formation but not EC mitogenesis. The discrepancy may be due to differences in the activity of PLF and PRL or heterogeneous responses of EC from different organ site origins.

The phorbol ester-induced expression of PLF requires AP-1 and Sph-1 sites in the promoter region (12), implicating the AP-1 family members Fra-1, JunB, and JunD as relevant transcription factors (14). AP-1-regulated PLF transcription is suppressed by glucocorticoid receptor activation (12). *In vivo*, GATA-2 and GATA-3 stimulate PLF transcription, and GATA-2-deficient placentas display significantly less proangiogenic activity. We now show that active STAT5 binds to the promoter region of PLF1 and induces transcription. In aggregate, these findings indicate a complex transcriptional regulation of PLF expression.

Once released, PLF binds to EC with high affinity (27). PLF binds to the insulin-like growth factor 2/mannose 6-phosphate receptor, which is required for PLF activity, but it is unclear how this receptor triggers the angiogenic signaling cascade (28). The 16-kDa PRL fragment competes with PLF for high affinity endothelial cell binding, suggesting that the PRL receptor may also be involved (29). Although the precise mechanism of receptor activation is unclear, it appears to involve a G protein-coupled pathway and mitogen-activated phospho-kinase (13). PRL family members rapidly activate JAK2 and consequently STAT1, STAT3, and STAT5 (30–33). The potential involvement of STAT5 upstream and downstream of PLF would set the stage for a positive feedback loop that could amplify and sustain proangiogenic signals.

Our findings are consistent with a model where FGF2 (and other proangiogenic stimulators) activates STAT5, which then induces the production of PLF. This secreted molecule then orchestrates EC migration, invasion, and tube formation. The apparent reliance of FGF2 on a secreted autocrine factor to exercise its full proangiogenic potential is provocative. However, this would be a biologically economical mechanism for integrating signals from several proangiogenic factors and synchronizing EC movement and differentiation in the interest of forming blood vessels in a rapid and efficient manner. The disruption of angiogenesis by interfering with PRL family member signaling could be an attractive therapeutic anti-tumor strategy but requires a better understanding of human PRL family members and isoforms in tumor angiogenesis.

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REFERENCES

1. Hanahan, D., and Folkman, J. (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86,** 353–364
2. Carmeliet, P., and Jain, R. K. (2000) Angiogenesis in cancer and other diseases. *Nature* **407,** 249–257
3. Eswarakumar, V. P., Lax, I., and Schlessinger, J. (2005) Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev.* **16,** 139–149
4. Yang, X., Qiao, D., Meyer, K., and Friedl, A. (2009) Signal transducers and activators of transcription mediate fibroblast growth factor-induced vascular endothelial morphogenesis. *Cancer Res.* **69,** 1668–1677
5. Deo, D. D., Axelrad, T. W., Robert, E. G., Marcheselli, V., Bazan, N. G., and Hunt, J. D. (2002) Phosphorylation of STAT-3 in response to basic fibroblast growth factor occurs through a mechanism involving platelet-activating factor, JAK-2, and Src in human umbilical vein endothelial cells. Evidence for a dual kinase mechanism. *J. Biol. Chem.* **277,** 21237–21245
6. Su, W. C., Kitagawa, M., Xue, N., Xie, B., Garofalo, S., Cho, J., Deng, C., Horton, W. A., and Fu, X. Y. (1997) Activation of Stat1 by mutant fibroblast growth-factor receptor in thanatophoric dysplasia type II dwarfism. *Nature* **386,** 288–292
7. Langley, R. R., Ramirez, K. M., Tsan, R. Z., Van Arsdall, M., Nilsson, M. B., and Fidler, I. J. (2003) Tissue-specific microvascular endothelial cell lines from H-2K(b)-tsA58 mice for studies of angiogenesis and metastasis. *Cancer Res.* **63,** 2971–2976
8. Irizarry, R. A., Bolstad, B. M., Collin, F., Cope, L. M., Hobbs, B., and Speed, T. P. (2003) Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res.* **31,** e15
9. Kendziorski, C. M., Newton, M. A., Lan, H., and Gould, M. N. (2003) On parametric empirical Bayes methods for comparing multiple groups using replicated gene expression profiles. *Stat. Med.* **22,** 3899–3914
10. Toft, D. J., Rosenberg, S. B., Bergers, G., Volpert, O., and Linzer, D. I. (2001) Reactivation of proliferin gene expression is associated with increased angiogenesis in a cell culture model of fibrosarcoma tumor progression. *Proc. Natl. Acad. Sci. U.S.A.* **98,** 13055–13059
11. Jackson, D., Volpert, O. V., Bouck, N., and Linzer, D. I. (1994) Stimulation and inhibition of angiogenesis by placental proliferin and proliferin-related protein. *Science* **266,** 1581–1584
12. Mordacq, J. C., and Linzer, D. I. (1989) Co-localization of elements required for phorbol ester stimulation and glucocorticoid repression of proliferin gene expression. *Genes Dev.* **3,** 760–769
13. Groskopf, J. C., Syu, L. J., Saltiel, A. R., and Linzer, D. I. (1997) Proliferin induces endothelial cell chemotaxis through a G protein-coupled, mitogen-activated protein kinase-dependent pathway. *Endocrinology* **138,** 2835–2840
14. Groskopf, J. C., and Linzer, D. I. (1994) Characterization of a delayed early serum response region. *Mol. Cell. Biol.* **14,** 6013–6020
15. Auerbach, R., Akhtar, N., Lewis, R. L., and Shinners, B. L. (2000) Angiogenesis assays. Problems and pitfalls. *Cancer Metastasis Rev.* **19,** 167–172
16. Kibbey, M. C., Grant, D. S., and Kleinman, H. K. (1992) Role of the SIKVAV site of laminin in promotion of angiogenesis and tumor growth. An *in vivo* Matrigel model. *J. Natl. Cancer Inst.* **84,** 1633–1638
17. Linzer, D. I., and Nathans, D. (1984) Nucleotide sequence of a growth-related mRNA encoding a member of the prolactin-growth hormone family. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 4255–4259
18. Linzer, D. I., Lee, S. J., Ogren, L., Talamantes, F., and Nathans, D. (1985) Identification of proliferin mRNA and protein in mouse placenta. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 4356–4359
19. Hemberger, M., Nozaki, T., Masutani, M., and Cross, J. C. (2003) Differential expression of angiogenic and vasodilatory factors by invasive trophoblast giant cells depending on depth of invasion. *Dev. Dyn.* **227,** 185–191
20. Fassett, J. T., Hamilton, R. T., and Nilsen-Hamilton, M. (2000) Mrp4, a new mitogen-regulated protein/proliferin gene; unique in this gene family for its expression in the adult mouse tail and ear. *Endocrinology* **141,** 1863–1871
21. Muscat, G. E., Gobius, K., and Emery, J. (1991) Proliferin, a prolactin/growth hormone-like peptide represses myogenic-specific transcription by the suppression of an essential serum response factor-like DNA-binding activity. *Mol. Endocrinol.* **5,** 802–814
22. Wang, J. W., Jiang, Y. N., Huang, C. Y., Huang, P. Y., Huang, M. C., Cheng, W. T., Shen, C. K., and Ju, Y. T. (2006) Proliferin enhances microvilli formation and cell growth of neuroblastoma cells. *Neurosci. Res.* **56,** 80–90
23. Peles, E., Lidar, Z., Simon, A. J., Grossman, R., Nass, D., and Ram, Z. (2004) Angiogenic factors in the cerebrospinal fluid of patients with astrocytic brain tumors. *Neurosurgery* **55,** 562–568
24. Kandel, J., Bossy-Wetzel, E., Radvanyi, F., Klagsbrun, M., Folkman, J., and Hanahan, D. (1991) Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. *Cell* **66,** 1095–1104
25. Wiemers, D. O., Shao, L. J., Ain, R., Dai, G., and Soares, M. J. (2003) The

mouse prolactin gene family locus. *Endocrinology* **144,** 313–325
26. Corbacho, A. M., Martínez De La Escalera, G., and Clapp, C. (2002) Roles of prolactin and related members of the prolactin/growth hormone/placental lactogen family in angiogenesis. *J. Endocrinol.* **173,** 219–238
27. Nelson, J. T., Rosenzweig, N., and Nilsen-Hamilton, M. (1995) Characterization of the mitogen-regulated protein (proliferin) receptor. *Endocrinology* **136,** 283–288
28. Volpert, O., Jackson, D., Bouck, N., and Linzer, D. I. (1996) The insulin-like growth factor II/mannose 6-phosphate receptor is required for proliferin-induced angiogenesis. *Endocrinology* **137,** 3871–3876
29. Clapp, C., and Weiner, R. I. (1992) A specific, high affinity, saturable binding site for the 16-kilodalton fragment of prolactin on capillary endothelial cells. *Endocrinology* **130,** 1380–1386
30. Brockman, J. L., Schroeder, M. D., and Schuler, L. A. (2002) PRL activates the cyclin D1 promoter via the Jak2/Stat pathway. *Mol. Endocrinol.* **16,** 774–784
31. DaSilva, L., Rui, H., Erwin, R. A., Howard, O. M., Kirken, R. A., Malabarba, M. G., Hackett, R. H., Larner, A. C., and Farrar, W. L. (1996) Prolactin recruits STAT1, STAT3 and STAT5 independent of conserved receptor tyrosines TYR402, TYR479, TYR515 and TYR580. *Mol. Cell. Endocrinol.* **117,** 131–140
32. Tourkine, N., Schindler, C., Larose, M., and Houdebine, L. M. (1995) Activation of STAT factors by prolactin, interferon-γ, growth hormones, and a tyrosine phosphatase inhibitor in rabbit primary mammary epithelial cells. *J. Biol. Chem.* **270,** 20952–20961
33. Welte, T., Garimorth, K., Philipp, S., and Doppler, W. (1994) Prolactin-dependent activation of a tyrosine phosphorylated DNA binding factor in mouse mammary epithelial cells. *Mol. Endocrinol.* **8,** 1091–1102