Supplemental Methods

Antibodies. Monoclonal antibodies against integrin αvβ5 (MAB1961Z) were purchased from Chemicon. Antibodies against β3 integrin (H-96; sc-14009), β5 integrin (H-96; sc-14010), ObR (H-300; sc-8325 and B-3; sc-8391), phospho-FAK (Tyr [Y] 861; sc-101679) and phosphotyrosines (PY20; sc-508) were obtained from Santa Cruz Biotechnology. Antibodies against phospho-FAK (Y397; #3283; and Y925; #3284) and FAK (#3285), phospho-JAK2 (Tyr1007/1008; #3771) and JAK2 (D2E12; #3230), phospho-Src (Y416; #2101), non-phospho-Src (Y527; #2107) and Src (32G6; #2123) were from Cell Signaling Technologies. Antibodies against human PTP1B (AF1366) were from R&D Systems, against GAPDH (BT46-9995-55) from Biotrend.

Cell Culture. Cells were isolated from peripheral human blood, cultivated and characterized according to an established protocol [1, 2]. They were CD31+, CD34−, CD45+, displayed limited proliferation ability, and did not form de novo blood vessels. Therefore, in accordance with a recently proposed definition [3], the cells used in the present study were termed circulating angiogenic cells (CAC). Analysis was performed after cultivation for 7 days, and cells were harvested with 5 mM EDTA in PBS (5 min, 37°C). Human umbilical vein endothelial cells (HUVEC; PromoCell) were cultured on gelatine-coated dishes in endothelial cell basal medium (EBM-MV; PromoCell) supplemented with 2% FCS, 5 ng/mL epidermal growth factor, 22.5 µg/mL heparin and 1 µg/mL hydrocortisone. Cells were harvested by trypsination, and used from passage 2 to 5.

Plasmid Transfection. CAC were transfected with either wild type (WT), constitutively active (CA; Y529F) or dominant negative (DN; K296R/Y528F) *Src* kinase cDNA-containing plasmids (all in pUSEamp; Upstate Biotechnology; and kind gift from T. Byzova) using Metafectene™ Pro transfection reagent (Biontex) and a DNA:transfection reagent ratio of 1:4,

optimized in preliminary experiments. Medium was changed 6 hours later, and cells were analyzed as described below.

Cell Labeling, Leptin Stimulation, and Specificity Experiments. For fluorescence labeling of CAC, cells were incubated with 2.5 µg/mL CM-DiI in serum-free medium for 10 minutes at 37°C and 10 minutes at 4°C. On day 6, CAC were washed with PBS and incubated for 24 hours with either recombinant human leptin or VEGF (both R&D Systems), or control (PBS), at final concentrations of 10 or 100 ng/mL for 24 hours. Specificity experiments were performed by incubation of cells with neutralizing antibodies against either leptin (anti-Ob; 100-fold molar excess) or the leptin receptor (anti-ObR; 10 µg/mL; both R&D Systems), or after transfection of CAC with human ObR siRNA (50 pmol per 6-well; sc-36115; Santa Cruz). Non-specific IgG antibodies (R&D Systems) or scrambled control siRNA (sc-37007) were used as negative control. Ligand binding to integrins was inhibited by RGD peptide (Gly-Arg-Gly-Asp-Ser; 250 µg/mL; G4391; Sigma), or monoclonal anti-αvβ5 antibodies (10 µg/mL; clone P1F6; Chemicon), and preincubation for 30 minutes at 37°C. Specific signal transduction pathways were inhibited by preincubation of CAC with 10, 25, or 50 µM of PTP1B inhibitor (Calbiochem), or with 10 µM of AG490 (Calbiochem), SU6656 (Sigma), or PP2 (Calbiochem). Equal concentrations of PP3 (Calbiochem) or volumes of DMSO (Sigma) were added as negative control.

***In Vitro* Angiogenesis Assays.** *Matrigel Tube Formation Assay*. CAC adhesion to vascular-like tubular structures was examined by co-incubation of $3x10^3$ CM-DiI-labeled CAC and $1.2x10^4$ HUVEC for 8 hours in 96-well plates precoated with 50 µL ECMatrix™ (Chemicon) and photographed on a fluorescence microscope (Zeiss Axiovert 200). The number of CM-DiI-positive cells adherent to tubular structures provided by HUVEC was counted in 8 random microscope fields and expressed as cells per mm tube length.

Spheroid Angiogenesis Assay. Fluorescence-labeled CAC ($8x10^3$) and HUVEC ($3.2x10^4$) were resuspended in 10 mL EBM-MV containing 20% methylcellulose solution (dissolved in M199 medium; Sigma) and incubated in round-bottom 96-well plates (100 µL per well) for 24 hours to form spheroids. Type I rat tail collagen (BD Biosciences) was diluted 1:1 with 0.1% acetic acid, mixed with 10X M199 medium, and neutralized with 0.2 N NaOH immediately before use. Spheroids were harvested in methylcellulose solution supplemented with 5% FCS and gently mixed (1:1) with collagen working solution. Spheroid suspensions were distributed into 24-well plates (1 mL per well) and incubated at 37°C for 30 minutes. After solidification of the collagen, medium supplemented with 4% FCS was added and incubated for 24 hours at 37°C. Pictures of 10 spheroids per variable at random fields were taken and evaluated using Zeiss AxioVision 3.1 software.

***In vivo* Angiogenesis Assays.** *Chorioallantoic Membrane Assay (CAM)*. Fertilized avian-leukosis-negative White Leghorn chicken eggs (Charles River Laboratories) were incubated in a rotating incubator at 38°C. On day 3, egg contents were transferred to sterile plastic weigh boats, covered with square Petri dishes and kept in a stationary incubator at 38°C and 85% humidity. For preparation of collagen onplants, eight volumes of rat tail collagen type I were neutralized with one volume of 10X Minimal Essential Medium (MEM) and one volume 0.1 M NaOH (pH 7.4), 2.5% BSA, and 0.25 M HEPES buffer were added. Then, $4x10^5$ CAC (in 200 µL EBM) were combined with 1 mL neutralized collagen, and 30 µL of the CAC/collagen mix (containing $1x10^4$ CAC) were placed on top of two layers of nylon mesh (Tetko) and allowed to polymerize at 37°C for 45 minutes. Following solidification, six onplants were grafted on top of the CAM of 10 day-old chicken embryos, employing six embryos per variable. Angiogenesis was scored after incubation for 65 to 72 hours using a Olympus stereomicroscope. Data are presented as number of grids containing newly formed blood vessels over the total number of grids ('angiogenic index').

Murine Hindlimb Ischemia Model. Ten to twelve week-old male athymic nude mice (NMRI-Foxn1$^{nu/nu}$) were obtained from Harlan Winkelmann. After anesthesia by intraperitoneal injection of xylazine / ketamine hydrochloride, unilateral hindlimb ischemia was induced by permanent ligation of the right femoral artery (immediately distal to the origin of the deep femoral artery) as well as the distal portion of the saphenous artery with 6-0 silk sutures. One day later, mice received CAC ($1x10^6$ cells) or an equal volume of PBS by intravenous injection. Before and immediately after the procedure, and during follow-up examinations on days 1, 2 and 4, mice were placed on 37°C heated pads, and limb perfusion was measured by laser Doppler imaging (PIM II, Perimed) as previously described [4,5]. Capillary density in the gastrocnemius muscle was assessed 10 days after induction of ischemia on 5 µm-thick, acetone-fixed frozen sections (200 µm apart) after staining with antibodies against CD31 (sc-18916; 1:50 dilution), followed by Cy3-labeled secondary antibodies (Molecular Probes). Cell nuclei were counterstained with DAPI. The number of CD31-immunopositive cells per muscle fiber was manually counted on 4 randomly selected microscope fields per section (200x magnification). All animal care and experimental procedures were approved by the State Board of Animal Welfare in accordance with national and institutional guidelines for the care and use of laboratory animals.

Immunoprecipitation, SDS Gel Electrophoresis and Western Blot Analysis. CAC were washed with ice-cold PBS, scraped off the culture plate, and resuspended in 100 µL lysis buffer (1% Triton-X 100, 150 mM NaCl, 50 mM TRIS, 5 mM EDTA, pH 7.5) containing fresh protease (4 mg/mL aprotinin, 4 µg/mL leupeptin, 4 µg/mL pepstatin A, 1 mM PMSF) and phosphatase (20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4O_7P_2$) inhibitors. After incubation for 20 minutes on ice, cell lysates were cleared by centrifugation at 13,000 rpm for 10 minutes at 4ºC. For immunoprecipitation (all steps performed at 4°C), 50-100 µg of total cell lysates were precleared with 50 µL nProtein A Sepharose™ 4 Fast Flow beads (GE Healthcare) in

500 µL lysis buffer for 2 hours under rotation. After centrifugation, supernatants were incubated with 2 µg antibody for 2 hours, 50 µg beads added, and the lysate-bead mixtures incubated under rotary agitation overnight. Equal amounts of protein were loaded and fractionated by SDS gel electrophoresis and transferred to nitrocellulose membranes (Protran®, Whatman). Membranes were blocked in 1% BSA (in TBS/0.1% Tween-20) for 2 hours prior to incubation with primary antibodies overnight at 4ºC. Visualization of protein bands was achieved using a HRP-conjugated secondary antibody (dilution, 1:3,000; Amersham Biosciences) for 2 hours, followed by detection of HRP with enhanced chemiluminescent substrate (Fisher Thermo Scientific).

Recruitment of Obese, Hyperleptinemic Individuals. Peripheral venous blood samples were collected from obese individuals (Body-Mass-Index, ≥35 kg/m^2) attending a professional weight reduction program (OPTIFAST-52®; Nestlé Health Care Nutrition GmbH) at the University of Goettingen. Individuals were recruited upon enrollment and re-examined after successful weight reduction, defined as body mass index ≤35 kg/m² and/or weight reduction of more than 10% of the initial body weight, 6 months later. Study participants were matched in terms of gender and age to normal-weight (BMI, 20-25 kg/m^2), healthy volunteers. The study protocol was approved by the institutional ethics review board at the University of Goettingen, and study participants gave written informed consent.

Statistical Analysis. Results are presented as mean ± SEM. Statistical analyses were performed using unpaired Student´s *t* test for comparisons between two means, or paired *t* test for comparisons before and after weight loss. A *P* value of less than 0.05 was considered statistically significant. Statistical analysis were performed using GraphPad Prism software, version 4.01.

References

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