Supporting Information

Coffman *et al.* 10.1073/pnas.0812010106

SI Text

Materials. Human umbilical vein endothelial cells and EGM-2 media were purchased from Lonza. Vitronectin, HKa, bFGF, DAPI, and 8-μm 24-well transwell inserts were purchased from BD Bioscience. Human spleen ferritin was purchased from Scripps Laboratory and was >98% pure by SDS/PAGE. CellTiter 96 AQ_{ueous} Nonradioactive Cell Proliferation Assay was purchased from Promega. QCM Chemotaxis 96-well cell migration assay kit, in vitro angiogenesis assay kit, and growth factor-reduced matrigel were purchased from Chemicon. Anti-cleaved caspase 3 was purchased from Cell Signaling. Anti-CD31 was purchased from BD Bioscience. Antismooth muscle actin was purchased from Biomeda. Casein from bovine milk purified protein was purchased from Sigma. Synthetic peptides were purchased from AnaSpec, San Jose, CA. Horseradish peroxidase-conjugated streptavidin was purchased from Pierce.

Solid Phase Binding Assays. The number of biotin molecules per HKa or D5 was determined using the EZ biotin quantification kit (Pierce). To perform the binding assay, microtiter plates (Nalge Nunc International, Rochester, NY) were coated with human spleen ferritin (2.5 μg/100 μL per well) in PBS overnight at 4°C. The plates were washed with PBST (PBS + 0.1% Tween 20) and blocked with 2% BSA. Biotinylated HKa or D5 at increasing concentrations was allowed to bind to the ferritin-coated wells for 2 h at 27°C. The wells were washed 3 times with PBST, treated with streptavidin-peroxidase for 1 h at 27°C, incubated with TMB substrate (3,3′, 5,5′-tetramethylbenzidine; Pierce), and the absorbance at 450 nm measured. To measure nonspecific binding, control wells were coated with 2.5 μg/100 μL native HK, and biotinylated HKa or D5 binding was analyzed in the same manner as above. To assess the amount of ferritin bound to the microtiter plate, biotinylated ferritin was adsorbed onto the microtiter plate in increasing amounts until saturation was reached. Maximal binding (Bmax) was determined through Scatchard analysis. To narrow the ferritin binding site, synthetic peptides were synthesized by AnaSpec, San Jose, CA. Six peptides were created and used as competitors at 10- and 50-fold molar excess (Table S1): peptide 1, ^{440}G-L^{473}; peptide 2, ^{474}D-K^{502}; peptide 2A, ^{474}D-K^{487}; peptide 2B, ^{481}H-G^{496}; peptide 2C, ^{488}H-K^{502}; peptide 3, ^{567}D-T^{591} (amino acid numbering excludes the 18-aa signal sequence). Nonbiotinylated HKa and casein were used as positive and negative control competitors, respectively.

Time-Lapse Microscopy. Six-well plates were coated with vitronectin (2 μg/ml in PBS) for 1 h at 27°C. HUVECs were seeded onto the coated plates at a density of 44,500 cells per ml in M199 media with 20 ng/ml bFGF and 10 μM $ZnCl_2$. Cells were allowed to adhere to the plate for 1 h then treated with 50 nM HKa with or without 50 nM human spleen ferritin, 50 nM ferritin alone, or a saline control. Cells were observed by using time-lapse microscopy for 24 h. The number of cells entering apoptosis, as defined by the appearance of the first apoptotic bleb, was counted over a 24-h period.

Caspase and DAPI Staining. Coverslips were coated with vitronectin (2 μg/ml in PBS) for 1 h at 27°C and placed within a 100-mm tissue culture dish. HUVECs were seeded onto the plates at a density of 30,000 cells per ml in M199 media with 10 ng/ml bFGF and 10 μM $ZnCl_2$. Cells were allowed to adhere for 1 h then treated with 10 nM HKa with or without 10 nM ferritin, 10 nM ferritin alone, or with a saline control. Cells on coverslips were incubated for 24 h, fixed with 10% formalin, washed in PBS, permeablized with 0.1% Trition X 100, washed, and incubated with rabbit anticleaved caspase 3 antibody followed by FITC linked goat anti-rabbit secondary antibody and DAPI. The number of cells positive for cleaved caspase 3 and fragmented nuclei (as identified through DAPI staining) was counted at 1000× magnification using a Zeiss Axioplan microscope.

Migration Assays. HUVECs in M199 media with 10 μM $ZnCl_2$ were seeded into the top chamber of a 24-well 8-μm transwell insert. Cells were treated with 50 nM HKa, 50 nM HKa + 50 nM Ft, 50 nM Ft, or saline control. M199 media with 10 μM $ZnCl_2$ and 10 ng/ml bFGF was added to the bottom chamber of the transwell insert. The cells were incubated for 5–6 h at 37°C, 5% CO_2. The transwell insert was removed and migration quantified using CyQuant GR. The control treatment value was considered 100% migration.

In Vitro Tube Formation. Ninety-six-well plates were coated with matrigel for 1 h. HUVECs in M199 media containing 10 ng/ml bFGF and 10 μM $ZnCl_2$ were seeded onto the wells of the in vitro angiogenesis assay kit (Chemicon) at a density of 33,000 cells per ml. The cells were treated with HKa (10 nM, 50 nM) and ferritin (10 nM, 50 nM), alone or together, and allowed to differentiate to form 2D tube-like structures on the matrigel surface. After 7 h the number of branch points per 100 viable cells was counted as a measure of endothelial cell differentiation. Cell viability was assessed through the addition of 20 μM calcein AM, incubation for 20 mins at 37°C, followed by fluorescent microscopy.

Effect of HKa and Ferritin on Tumor Angiogenesis. Four million PC3 prostate cancer cells in a 1:1 volume ratio with growth factor-reduced matrigel mixed with 400 nM HKa, 400 nM HKa + 400 nM Ft, 400 nM Ft, or saline control was injected s.c. in the flank of athymic nu/nu mice. Each of 12 mice received 1 tumor per flank. In total, 9 control tumors, 6 HKa tumors, 6 HKa + Ft tumors, and 3 Ft tumors were injected. Tumors were resected 10 days postinoculation and tumor size measured with a caliper. Tumors were cryosectioned and stained with anti-CD-31 to identify endothelial cells in blood vessels. Vessel density was assessed as described by Weidner (1). Briefly, tumors were analyzed under 100× magnification and vessel hot spots (areas containing blood vessels) were identified and counted throughout the entire tumor cross-section. The density of hot spots per mm^2 tumor cross-sectional area was calculated. The individual hot spots were examined at 400× magnification and the number of blood vessels per field of view counted to calculate mean blood vessel density. Two sections per tumor were analyzed.

Statistical Analysis. For analysis of survival over time, a 2-way ANOVA model was fitted to include factors for time, group, and the time by group interaction to see whether the rate of change

in groups was different. If the interaction was significant, then additional testing was performed to determine which group(s) was different and at what point(s) in time the differences became statistically significant.

1. Weidner N, Semple JP, Welch WR, Folkman J (1991) Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. *N Engl J Med* 324:1–8.

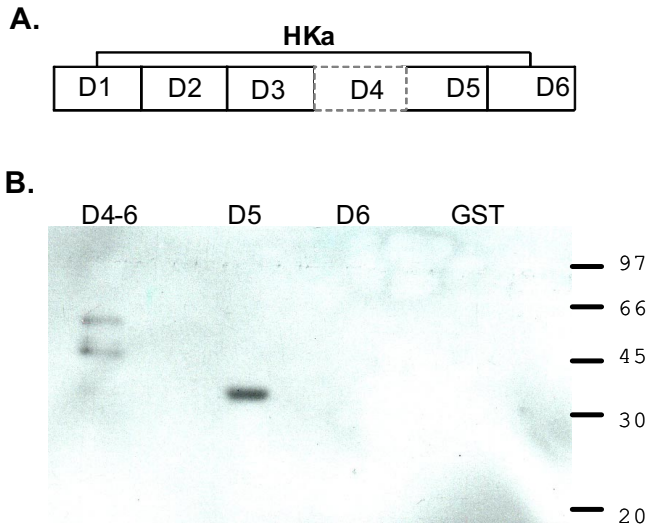

Fig. S1. Ferritin binds to domain 5 (D5) of HK/HKa. (*A*) Schematic of HK/HKa domains. (*B*) Ferritin ligand blot of recombinant D4-D6-GST, D5-GST, D6-GST, and a GST control.

Table S1. Peptides used in competition experiments

Peptide	Sequence
Peptide 1	^{440}GHGLGHGHEQQHGLGHGHKFKLDDDLEHQGGHVL^{473}
Peptide 2	^{474}DHGHKHKHGHGHGKHKNKGKKNGKHNGWK^{502}
Peptide 2A (N-terminal region of peptide 2)	^{474}DHGHKHKHGHGHGK^{487}
Peptide 2B (central region of peptide 2)	^{481}HGHGHGKHKNKGKKNG^{496}
Peptide 2C (C-terminal region of peptide 2)	^{488}HKNKGKKNGKHNGWK^{502}
Peptide 3	^{567}DDWIPDIQTDPNGLSFNPISDFPDT^{591}

Sequence and map position of HK peptides used as competitors in solid phase binding assays. Amino acid numbering excludes the 18-amino-acid signal sequence.