Supplementary Figure S1

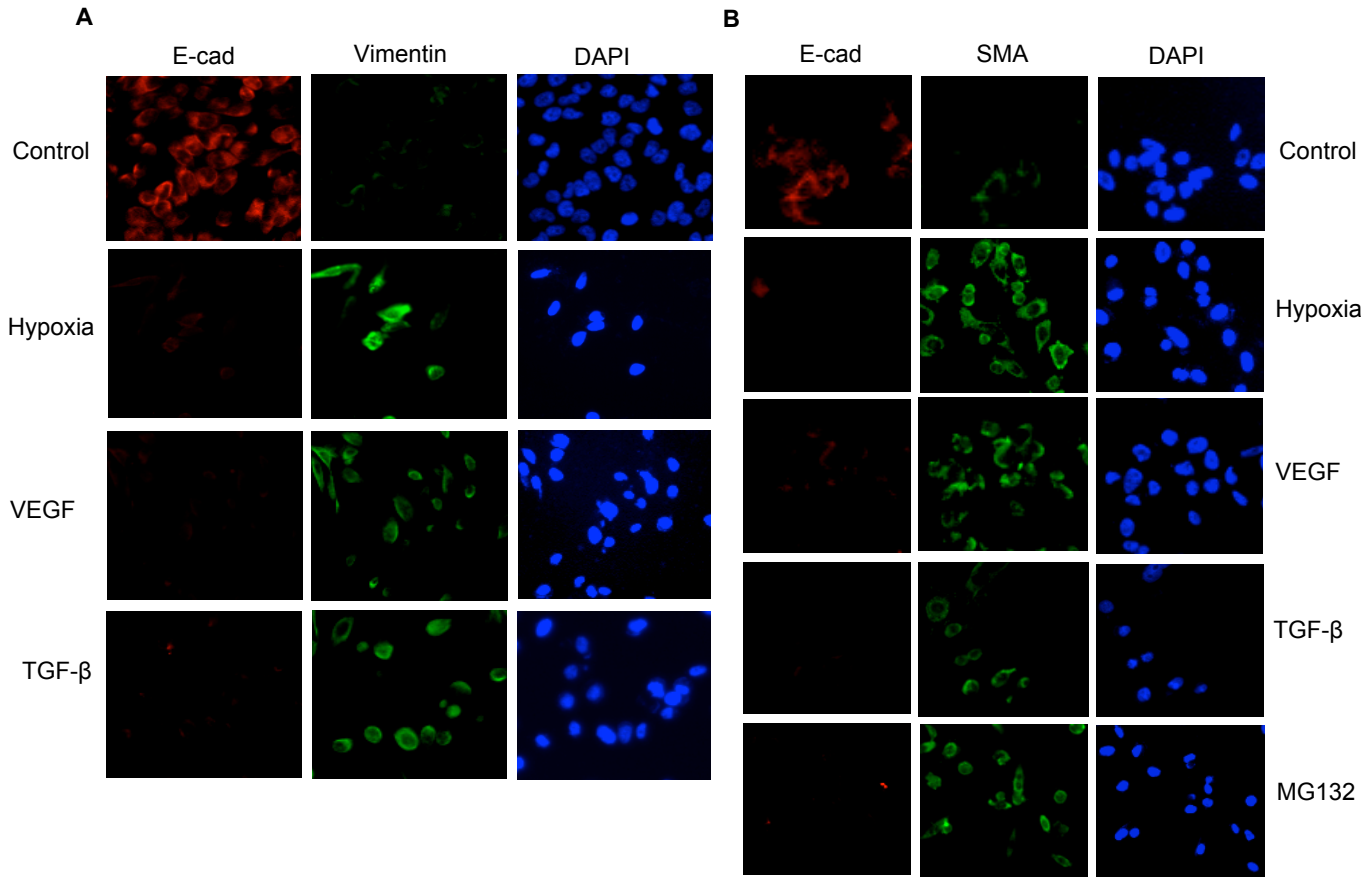

Figure S1 Immunofluorscence Analysis of EMT in PCa Cells. See Fig. 1. PC3 cells were treated with EMT-inducing stimuli (hypoxia, VEGF, TGF-β or MG132) as described for Figures 1, 4 and 5 and processed for immunofluorescence microscopy as described in Supplementary Experimental Procedures. Figure S1A: E-cadherin and vimentin staining; Figure S1B: E-cadherin and α-smooth muscle actin staining. DAPI staining reveals nuclei. Note that the EMT stimuli diminish E-cadherin staining and induce staining of the mesenchymal markers vimentin and α-smooth muscle actin. Original magnification of all photomicrographs is 10X.

Supplementary Experimental Procedures

Analysis of clinical specimens. Archival PCa specimens of defined Gleason grades were procured as described in Experimental Procedures. These archival collections were from a patient population that ranged in age from 52- 75 (mean=64). None of the patients had received any hormonal or radiation therapy prior to radical prostatectomy or core biopsy. The collected archived samples were composed of 15% that had a total Gleason grade score of 6. Seventy-five percent of the specimens were classified as having a total Gleason grade of 7 in which 80 % of these had predominant Gleason grade 3 while grade 4 PCa was the major component in the other 20% of cases. The remaining cases (18%) were classified as Gleason grade 8-10.

Thirty cases of defined Gleason grades were selected for IHC analysis by a pathologist (I. Leav) from the archived collections referred to above. With the exception of 3 core biopsies, 27 samples were from patients that had undergone radical prostatectomy. Twenty of the selected cases had total Gleason scores of 6-7 and 10 had Gleason scores of 8-10 and all of these latter cases contained foci of grade 5 PCa. Twelve cases out of the 20 grade 3 PCa and 5 cases out of the 10 grade 5 PCa were from radical tissue specimens and the remaining cases were contained in the TMA.

Adhesive–coated glass slides of paraffin embedded specimens were stained with Abs specific for ERβ1 (1:200) (14C8;Gene Tex), E-cadherin (1:200) (4A2C7; Abcam) and Snail1 (1:500) (17732; Abcam), VEGF-A (1:200) (A20; Santa Cruz) and HIF-1α (1:200) (HIF-1α 67; Novus) as previously described (Leav, 2001). Human renal cell carcinoma served as a positive control for HIF-α and a previously documented highly immunopositive sample of grade 5 PCa was used as a positive control for Snail1 and VEGF-A. The scoring system used was a semi-quantitative method as described (Nanni et al., 2009) that is based upon the proportion of tumor cells stained quantity (q) and the staining intensity (I) to obtain a final score (Q) defined as the product of I X Q. The scoring system for q was: 0=negative, 1 =1-9 % positive cells, 2 =10-39% positive, 3= 40-69% positive, 4= 70-100% positive cells.. The scoring system for I was: 0 = negative, 1= low, 2= moderate, 3= intense immunostaining. All scoring was performed by a pathologist (I. Leav).

Immunofluorescence microscopy: PC3 cells were cultured on glass cover slips until sub-confluence was reached, treated with the stimuli described in the legend to Figure S1, fixed in paraformaldehyde (4%) and permeabilized with Triton-X-100 (0.1%). The coverslips were then incubated in PBS containing 10% goat serum with the following primary Abs: E-cadherin and vimentin (Dako), and α-smooth muscle actin (Cell Signaling). After washing with PBS, coverslips were incubated with fluorescein isothiocynate (FITC), or rhodamine-conjugated secondary Abs (Jackson Immunoresearch) for 1 h. The coverslips were mounted on slides with SlowFade Antifade containing DAPI and images were taken using an Olympus 1x71 fluorescence microscope.

PCR: RT-PCR was performed using Platinum *Taq* polymerase (Invitrogen) with Snail1-specific primers (Forward: 5'-GAAAGGCCTTCAACTGCAAA; Reverse: 5'-TGACATCTGAGTGGGTCTGG) or E-cadherin specific primers (Forward: 5'-CAGCGTCAACTGGACCATTG; Reverse: 5'-CCACCGTTCTCCTCCGTAGA). The forward and reverse primers for β-actin have been described previously (Mak et al., 2006). To quantify E-cadherin and vimentin expression, qPCR was conducted using TaqMan Gene Expression Assays (Applied Biosystems) with primers: CDH1 (Hs00170423_m1), Vim (Hs00185584_m1) and GAPDH (H299999905_m1). To quantify VEGF expression, qPCR was conducted using the Power SyBr Green PCR Master Mix (Applied Biosystems) with the primers: PGK1 (GGGCAAGGATGTTCTGTTCT, TCTCCAGCAGGATGACAGAC) and VEGF-A 165 (CGAAGTGGTGAAGTTCATGG, AAGATGTCCACCAGGGTCTC).

Mutational Analysis of the VEGF-A Promoter: The VEGF-A promoter was PCR amplified from human genomic DNA using KOD Hot Start DNA Polymerase (Novagen; Cat # 71086-3) with primers 5'-GCGAATTCCTCGAGGAGACAGGACTAGTGCACGAATGA-3' and 5'-CTGCAGAGAAGCTTGGTGTCTGTCTGTCTGTCCGTCAG-3'. PCR amplified fragment was confirmed by restriction mapping and cloned at Xho1-Hind III site into the pGL3 basic vector from Promega. The cloned VEGF promoter was sequenced to confirm the presence of the ERE and HRE. For mutational analysis, the ERE element (AA**TCA**GAC**TGA**CT) was mutated (AA**CTG**GAC**CAA**CT) using Quick Change XL Site Directed Mutagenesis Kit (Stratagene; Cat # 200517) and a pair or of following

primers; 5’-CTGGCAAGATCTGGGTGGATAACTGGACCAACTGGTCCCACTCTTCCCACAGG-3’ and 5’-CCTGTGGGAAGAGTGGGACCAGTTGGTCCAGTTATCCACCCAGATCTTGCCAG-3’.

Concurrently, the HRE sequence (T**ACGT**G) was mutated (T**AAAA**G) to generate a double mutant using the following primers: 5'-CCAGACTCCACAGTGCATAAAAGGGCTCCAACAGGTCCTC-3' and 5'-GAGGACCTGTTGGAGCCCTTTTATGCACTGTGGAGTCTGG-3'. The mutations were confirmed by sequencing the human VEGF promoter.