Angiostatin Inhibits Endothelial MMP-2 and MMP-14 Expression: A Hypoxia Specific Mechanism of Action

A Radziwon-Balicka[a,c], C Ramer[a], C Moncada de la Rosa[a,c], B Zielnik-Drabik[a], P Jurasz[a,b,c]

[a]*Faculty of Pharmacy and Pharmaceutical Sciences;* [b]*Department of Pharmacology, Faculty of Medicine and Dentistry; and* [c]*Cardiovascular Research Centre, University of Alberta, Edmonton, Alberta, Canada, T6G 2E1.*

Correspondence: Paul Jurasz, Faculty of Pharmacy and Pharmaceutical Sciences, 3-142E Katz Group-Rexall Centre for Pharmacy & Health Research, University of Alberta, Edmonton, AB, Canada, T6G 2E1. Tel: 780-492-2120; Fax: 780-492-1843; email: pjurasz@pharmacy.ualberta.ca.

Abstract

Angiostatin is an angiogenesis inhibitor in part generated by and released from platelets. Since platelets upon thrombus formation can give rise to areas of hypoxia, we investigated the effects of angiostatin on endothelial cell migration and apoptosis during hypoxia. Human microvascular endothelial cells (HMVEC-L) were exposed to angiostatin under normoxic or hypoxic conditions. Apoptosis was measured by flow-cytometry. HMVEC-L migration was studied using a modified Boyden Chamber assay, in which migration is MMP-dependent. MMP-2, MMP-14, and VEGF levels were measured using immunoblot, Q-PCR and ELISA. During hypoxia HMVEC-L were protected from angiostatin-induced apoptosis due to increased hypoxia-induced VEGF expression. However, MMP-dependent migration of HMVEC-L was inhibited by angiostatin under hypoxic but not normoxic conditions. Angiostatin decreased MMP-2 at the gene and protein levels only in HMVEC-L exposed to hypoxia. A similar result was obtained for MMP-14. Higher angiostatin concentrations, as would be seen during thrombosis, induced HMVEC-L apoptosis, which was not rescued by VEGF. Under hypoxic conditions angiostatin's primary anti-angiogenic mechanism is likely inhibition of endothelial cell MMP-dependent endothelial cell migration. Only at higher concentrations does angiostatin induce endothelial cell death. This study identifies a novel angiostatin anti-angiogenesis mechanism that is only triggered under pathological-like conditions.

Key words

Angiostatin, Hypoxia, Endothelial cells, Platelets, Matrix metalloproteinases

Abbreviations

Angst, angiostatin; BCECF AM, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethy ester; HMVEC-L, human microvascular endothelial cells derived from lung; K, kingle; MMP, matrix metalloproteinase; siRNA, short interfering RNA; VEGF, vascular endothelial growth factor

1. Introduction

Angiostatin is an angiogenesis inhibitor first discovered in a mouse Lewis Lung carcinoma model of concomitant resistance (O'Reilly et al., 1994). Angiostatin is formed by proteolysis of plasminogen and contains the first four-kringle subunits (K1-4). Subsequently, it has been shown that angiostatin is abundant in the plasma of healthy individuals and that it is constitutively generated by human platelets and released in active form upon aggregation (Jurasz et al., 2003a; Jurasz et al., 2006; Jurasz et al., 2010). In addition to platelets, angiostatin and other angiostatins (angiostatins containing K1-3, K4.5, and K1-5) have been shown to be generated by macrophages (Falcone et al., 1998) or various cancer cells (Soff, 2000) also through proteolytic means.

The exact mechanisms by which angiostatin inhibits angiogenesis remain enigmatic. Several groups have reported that angiostatin(s) inhibit endothelial cell proliferation by inducing cell cycle arrest (Griscelli et al., 1998; Meena et al., 2004), inhibit endothelial migration (Gately et al., 1996; Ji et al., 1998; Troyanovsky et al., 2001), and/or promote endothelial apoptosis (Claesson-Welsh et al., 1998; Hanford et al., 2003; Veitonmaki et al., 2004). One of the best-characterized angiostatin targets, which may mediate these anti-angiogenesis effects, is an ectopic ATP synthase on the endothelial cell surface (Wahl et al., 2005; Moser et al., 1999). Binding and inhibition of this extracellular ATP synthase by angiostatin K1-5 induces endothelial cell apoptosis (Veitonmaki et al., 2004). Furthermore, it has been postulated that the extracellular pH (pH_e) of an endothelial cell's microenvironment influences whether ATP synthase inhibition by angiostatin leads to an endothelial cell's demise (Wahl et al., 2005).

Indeed, at low tumor-like extracellular pH (6.7-6.9) angiostatin potentiates endothelial cell death (Wahl et al., 2002a; Wahl et al. 2002b; Jurasz et al., 2006), an effect attributed, at least in part, to the inhibition of the ATP synthase proton pump resulting in intracellular acidosis of the endothelial cell. As a result, angiostatin seemingly displays specificity for the tumor vasculature (Wahl et al., 2002b), and may partially explain why it has been investigated primarily in the context of tumor angiogenesis

Recently, angiostatin has been shown to be more than a tumor-specific angiogenesis inhibitor. Several studies suggest angiostatin may play a pathological role in cardiovascular diseases (Chung et al., 2006; Jurasz et al., 2010; Matsunaga et al., 2005; Sodha et al., 2009), as angiostatin is reported to negatively correlate with collateral vessel growth in patients with coronary artery disease (Matsunaga et al., 2005; Sodha et al., 2009). A common pathological feature between coronary artery disease and tumors is hypoxia, which lowers pH_e due to cellular anaerobic metabolism. Although the effect of decreased pH_e on angiostatin-induced endothelial cell death has been investigated, very little is known of how hypoxia influences angiostatin's mechanism of action. In addition to lowering pH_e, hypoxia also up-regulates vascular endothelial growth factor (VEGF) expression (Keshet et al., 1992). VEGF is a potent endothelial survival factor (Jurasz et al., 2011), which may antagonize angiostatin's anti-angiogenic effects (Chen et al., 2003). Hence, under hypoxic conditions when pH_e and VEGF signalling have opposing effects, angiostatin may promote anti-angiogenesis mechanisms other than endothelial cell death. One such alternate mechanism may be to disrupt endothelial matrix metalloproteinase (MMP) production, as previously we have demonstrated that platelet-generated angiostatin inhibits MMP-2 release from

endothelial cells under conditions of low pH_e (Jurasz et al., 2006). Although angiostatin production by MMPs has been widely studied, almost nothing is known about how angiostatin impacts endothelial cell MMP production. MMPs such as MMP-2 and MMP-14 play an important role remodelling a blood vessel's basement membrane and the extracellular matrix thus facilitating endothelial cell migration, an early stage of angiogenesis. Hence, the aim of our present study was to investigate the effects of angiostatin on endothelial cells under hypoxic conditions focusing primarily on angiostatin's pro-apoptotic and anti-MMP effects.

2. Materials and Methods

2.1 Reagents: Human plasma-isolated angiostatin was obtained from Pierce Biotechnology (Rockford, IL, USA). Recombinant $VEGF_{165}$ was obtained from R&D Systems (Minneapolis, MN, USA). 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethy ester (BCECF AM) was obtained from Invitrogen (Burlington, ONT, Canada). p53 activator II (RI-TATp53C′-WT) and activator III (2,5-bis-(5-Hydroxymethyl-2-thienyl)-furan) were obtained from Calbiochem (Mississauga, ONT, Canada).Unless otherwise specified all other reagents were obtained from Sigma (Mississauga, ONT, Canada).

2.2 Cell culture and Hypoxia: Human microvascular endothelial cells from lung (HMVEC-L) were obtained from Lonza (Walkersville, MD, USA) and cultured in a humidified atmosphere at 37^{o}C and 5% CO_2 in EGM-2 MV. Depending on the donor, the primary endothelial cells were serum starved for 4-16 hours in EBM-2 and 0.5% FBS prior to exposure to hypoxic conditions. The time of serum starvation prior to treatment was titrated to induce approximately 40% apoptosis following treatment under normoxic conditions. Hypoxia was induced by exposing endothelial cells for 48 hours under serum-starved conditions in a Billups-Rothenberg chamber continuously gassed with 95% N_2-5% CO_2.

2.3 Flow cytometry: Flow cytometry was performed to assess HMVEC-L- apoptosis as described previously using annexin V-fluorescein isothiocyanate (BD Biosciences, CA) and propidium iodide (Jurasz et al., 2011; Jurasz et al., 2006). Fluorescence was induced with an argon laser and detected on FL1 (525 nm BP filter) and FL3 (620 nm SP filter) on a Beckman Coulter Cytomics FC500 or Quanta SC flow cytometers, 10,000

events per sample. Compensation was performed using CYTOMICS^{TM} CXP or Cell Lab Quanta analysis software to account for fluorophore spectral overlap. Further apoptosis measurements were performed by analyzing cell cycle sub-diploid peak formation as described previously (Jurasz et al., 2011).

2.4 Intracellular pH measurements: HMVEC-L intracellular pH was measured using flow cytometry with the fluorochrome BCECF-AM (1 μM) and the green: red fluorescence ratios were determined as described by Cherlet and colleagues (Cherlet et al., 1999). The ratios were compared to pH calibration curves generated by incubating BCECF-AM loaded HMVEC-Ls in high-[K^+] buffers ranging from pH 6.8 to 7.6 (0.2 unit increments) in the presence of nigericin (10 μM) which allows for the equilibration of pH_i and pH_e.

2.5 Extracellular pH measurements: Following 48 hours incubation of HMVEC-L under normoxic or hypoxic conditions, the EBM-2 pH_e was immediately measured by an Accumet AB15 pH meter (Fisher Scientific, Ottawa, Canada).

2.6 ELISA: An active p53 ELISA (R&D Systems; Minneapolis, MN, USA) was performed on endothelial cells treated with 3 μM activator II and activator III according to manufacturer instructions. The results were expressed as arbitrary units of active p53 per mg of endothelial cell nuclear protein.

A VEGF ELISA (R&D Systems; Minneapolis, MN, USA) was performed to quantify endothelial soluble $VEGF_{165}$ production and VEGF release by human platelets (Jurasz et al., 2010).

2.7 siRNA Experiments: Transfection media, reagent, scrambled siRNA, VEGF, and MMP-2 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). P53

endo-ribonuclease siRNA (esiRNA) was obtained from Sigma. esiRNA is a pool of siRNAs obtained from cleavage of long double-stranded RNA (dsRNA) with *E. coli* RNase III. VEGF siRNA (60 nM), MMP-2 siRNA (90 nM) and p53 esiRNA (60 nM) were performed as described previously (Jurasz et al., 2011).

2.8 ATP Measurement by chemiluminescence: Extracellular ATP generated by HMVEC-L was measured using a lumi-aggregometer (Chronolog, Havertown, PA, USA) (Chung et al., 2002). Following 48 hours treatment under normoxic or hypoxic conditions, EBM-2 was removed from flasks and was incubated with luciferin-luciferase reagent (440 luciferase units ml^{-1} and 4 mg ml^{-1} of luciferin) (Chronolog) for 3 min at 37^{o}C in order for the ATP to react with luciferin-luciferase and generate chemiluminescence. To quantify extracellular ATP generation, standard curves were constructed from ATP standard (Chronolog) added to fresh EBM-2. To account for the effects of pH on enzyme kinetics of chemiluminescence generating proteins, two standard curves were generated. A standard curve with EBM-2 at pH 7.4 was used to quantify ATP generation by normoxic HMVEC-L, while a standard curve with EBM-2 formulated to pH 6.9 was used to quantify ATP generation by hypoxic HMVEC-L.

2.9 Reverse Transcriptase and Real Time-PCR: Total HMVEC-L RNA was isolated using the PARIS^{TM} protein and RNA isolation system (Applied Biosystems, Carlsbad, CA, USA). The RNA was reverse transcribed using Sensiscript reverse transcriptase (Qiagen, Mississauga, ON, Canada). Thereafter, quantitative real-time PCR was performed using SYBR GreenI PCR Master Mix and the ABI PRISM 7900HT (Applied Biosystems Inc., Foster City, CA) as described previously (Jurasz et al., 2011). 2 ng of transcribed DNA was used for each qPCR reaction in 20 μl using 0.3 μM of MMP-2

primer pairs (Forward-GAT GGA TAC CCC TTT GAC GGT and Reverse-GCT GTT GTA CTC CTT GCC ATT G) or RPL-32 (ribosomal protein L-32) primer pairs (Forward-TGC CCA ACA TTG GTT ATG GA and Reverse-TGG GGT TGG TGA CTC TGA TG) as an endogenous control. We used the relative quantification standard curve method to calculate the amount of mRNA normalized to RPL-32. Expected PCR product size was confirmed by standard reverse transcriptase-PCR.

2.10 Immunoblot: Immunoblots of HMVEC-L lysates were performed as previously described (Jurasz et al., 2003a). Blots were blocked overnight and then incubated with either goat anti-human MMP-2 (0.2 μg/ml), anti-human angiostatin (0.8 μg/ml) (R&D Systems), mouse anti-human $VEGF_{165}$ antibodies (1 μg/ml) (R&D Systems), mouse anti-human p53 antibodies (0.1 μg/ml) (Calbiochem), rabbit anti-human MMP-14 (MT1-MMP) (1 μg/ml) (Millipore) (Etobicoke, ONT, Canada), or mouse anti-eNOS (1:1000) (Abcam) (Toronto, ONT, Canada) for 2 hours. Anti-goat (0.2 μg/ml), anti-mouse (1:10000), or anti-rabbit (1:5000) horseradish peroxidase–conjugated antibodies were used as the secondary antibodies (Sigma, Oakville, ON, Canada). Immunoreactive bands were visualized with ECL Plus (Amersham Biosciences, San Francisco, CA). Subsequently, membranes were stripped and probed for β-actin with a β-actin-horseradish peroxidase–conjugated antibody (1:20000) (Sigma), which was used as a loading control. Blot bands were quantified using a VersaDoc MP5000 molecular imager with Quantity One software (Bio-Rad, Mississauga, ON, Canada) and expressed as arbitrary units of density per mg protein. HT-1080 fibrosarcoma conditioned medium containing pro-MMP-2 (72 KDa) was used as standard (Jurasz et al., 2001).

2.11 Gelatin zymography: Gelatin zymography was used to detect MMP-2 activity in HMVEC-L lysates. Zymography was performed using 8% SDS-PAGE with copolymerized gelatin (2 mg/ml) as described previously (Jurasz et al., 2003b; Jurasz et al., 2001; Jurasz et al., 2006). Conditioned medium from PMA-treated HT-1080 fibrosarcoma containing activated MMP-2 (64 KDa) was used as standard.

2.12 MMP-dependent Endothelial Cell Migration Assays: Endothelial cell migration assays were performed in duplicate as described previously (Yacyshyn et al., 2009), but with the following modifications. Cell culture inserts with 8 μm pores (BD Falcon^{TM}) were coated with gelatin (1 mg/ml) (Sigma) for 2 hours at 37^{o}C. After 2 hours, excess gelatin was removed, the inserts were washed 1x with 100 μl of sterile PBS, and allowed to air dry for 30 min.

Following 48 hours of exposure to normoxic or hypoxic conditions, HMVEC-L remaining attached to flasks were detached using 1ml of Trypsin-EDTA solution (Lonza). The trypsin was inactivated using 10 ml of EBM-2 + 0.5% FBS and HMVEC-L pelleted. The cells were washed 1x and resuspended in EBM-2 + 0.5% BSA, and counted. 1 x 10^5 HMVEC-L in 500 μl were added to each insert and allowed to migrate toward a VEGF (5 ng/ml) gradient formed by adding recombinant $VEGF_{165}$ to the wells of insert companion plates. Plates and inserts were incubated at 37°C in a humidified atmosphere with 5% CO_2 under normoxic conditions. After 24 hours, non-migrating HMVEC-L were removed by scrubbing the upper surface of the inserts with a cotton-tipped swab. The cells on the lower surface membrane were fixed in 4% formaldehyde in PBS and stained with Diff-Quik stain. The membrane was examined by light microscopy using an Olympus CKX41 microscope (Olympus America Inc., Melville, NY)

equipped with an *Infinity 1* digital camera. Photomicrographs were captured at the top, bottom, right, left, and center of each insert. Each field of view was counted using ImageJ software, and the results were expressed as the average number of HMVEC-L migrated per field of view.

2.13 Blood platelets and platelet aggregation: Approval for the current study was obtained from the University of Alberta Human Research Ethics Board, and the study was carried out in accordance with the Declaration of Helsinki. Blood was collected from healthy volunteers who had not taken any drugs for 14 days prior to the study. Platelet-rich plasma (PRP) was prepared as described previously (Jurasz et al., 2010). Alternatively, prostacyclin-washed platelets were prepared as described previously (Jurasz et al., 2003a; Jurasz et al., 2006). Platelet samples were pre-incubated for 2 minutes at 37°C in a lumi-aggregometer (Chronolog, Havertown, PA). Platelet aggregation was initiated by collagen (10 μg/ml) and monitored by Aggro-Link software for 6 minutes as previously described (Jurasz et al., 2003a). After aggregation, platelet pellets were separated from releasates using centrifugation (1000 *g* for 10 minutes) and stored at -80°C until assayed for angiostatin immunoblot or VEGF ELISA analysis (Jurasz et al., 2010).

2.14 Statistics: Statistics were performed using Graph Pad Prism 3.0 software. All means are reported with SE. One-way ANOVA with Tukey's multiple comparisons test and paired Student's T-tests were performed where appropriate. A P-value less than 0.05 was considered as significant.

3. Results

3.1 Hypoxia Protects Endothelial Cells from Angiostatin-induced Apoptosis

Under normoxic conditions incubation of serum starved HMVEC-L with physiological concentrations of angiostatin (30 μg/ml) did not promote endothelial cell apoptosis compared to control (40.5 ± 2.9 % vs. 40.4 ± 3.1%; *P > 0.05*) (Figure 1A and B). Under hypoxic conditions, apoptosis of serum-starved HMVEC-L decreased compared to normoxic controls irrespective of whether HMVEC-L were incubated without or with angiostatin (40.5 ± 2.9 % vs. 24.4 ± 3.6% vs. 28.8 ± 4.1%, respectively; *P < 0.05*). During hypoxia, angiostatin (30 μg/ml) failed to significantly increase HMVEC-L apoptosis compared to hypoxic controls (28.8 ± 4.1% vs. 24.4 ± 3.6%; *P > 0.05*) (Figure 1A and B). We further confirmed these apoptosis results by analyzing cell cycle subdiploid peak formation (online supplementary Figure 1).

Since others and we have previously shown that angiostatin potently promotes endothelial apoptosis (3-5 fold) under conditions of low pH_e (Jurasz et al., 2006; Wahl et al., 2002a), as would be generated during hypoxia, we tested whether hypoxia lowered pH_e in our experimental system. Compared to normoxic cells the pH of the medium bathing hypoxic HMVEC-L decreased significantly irrespective of the presence of angiostatin (7.44 ± 0.02 and 7.46 ± 0.05 vs. 6.97 ± 0.06 and 6.94 ± 0.06; *P < 0.05*) (Figure 1C). Hence, angiostatin failed to significantly increase HMVEC-L apoptosis in spite of the fact that the extracellular pH significantly decreased during hypoxia. Under normoxic conditions angiostatin lowered the HMVEC-L intracellular pH (pH_i) as compared to hypoxic controls cells which retained a physiological pH (6.93 ± 0.17 and

7.42 ± 0.13; *P < 0.05*); however, angiostatin did not produce a significant reduction in pH_i under hypoxic conditions (Figure 1D).

Since angiostatin did not significantly induce HMVEC-L apoptosis under hypoxic conditions even though pH_e significantly decreased, we examined whether plasma-derived angiostatin is functional by measuring its ability to inhibit one of its known endothelial targets, the extracellular ATP synthase (Moser et al., 1999). Under hypoxic conditions, angiostatin (30 μg/ml) was most effective at inhibiting extracellular ATP production by HMVEC-L compared to normoxic controls (0.7 ± 0.2 vs. 2.2 ± 0.4 nM; *P < 0.05*) (Figure 2A). However, at the same time, production of the potent endothelial survival factor VEGF increased from undetectable levels in the cellular medium during normoxia to over 110 pg/ml during hypoxia (Figure 2B). SiRNA-mediated knockdown of VEGF expression by hypoxic HMVEC -L resulted in a significant increase in endothelial apoptosis in response to angiostatin (30 μg/ml) (Figure 2C); thus, confirming VEGF's protective and antagonistic role (Chen et al., 2003) against angiostatin-mediated apoptosis.

3.2 Angiostatin Inhibits MMP-2 Expression by Hypoxic Endothelial Cells

Since inducing apoptosis may not be angiostatin's main mechanism of action during hypoxia, we investigated its effect on MMP-2, a molecular mediator of angiogenesis (Jurasz et al., 2006). Incubation of HMVEC -L under hypoxic conditions in the presence of angiostatin (30 μg/ml) resulted in a significant reduction in MMP-2 mRNA compared with control hypoxic HMVEC-L (0.79 ± 0.16 fold change vs. 1.40 ± 0.12 fold change; *P < 0.05*), which tended to have higher amounts of MMP-2 mRNA relative to normoxic cells

(Figure 3A). Consequently, compared to normoxic HMVEC-L hypoxic endothelial cells exposed to angiostatin generated significantly less 72 KDa MMP-2 protein (pro form) as measured in HMVEC-L lysates by gelatin zymography (4.10 ± 0.50 vs. 2.64 ± 0.16 arbitrary unit of density/mg protein; $P < 0.05$) and immunoblot (2.31 ± 0.42 vs. 0.83 ± 0.38 arbitrary unit of density/mg protein; $P < 0.05$) (Figure 3B and C). Similarly, angiostatin treatment of HMVEC-L under hypoxic conditions resulted in a loss of 64 KDa (activated form) of MMP-2 (Figure 3B and C). We further investigated whether MMP-14 (MT1-MMP), which converts 72 KDa pro-MMP-2 into 64 KDa active MMP-2, is affected by angiostatin during hypoxia. We found that under hypoxic conditions angiostatin significantly reduced total (pro- and active form) HMVEC-L MT1-MMP levels (Figure 4A).

To investigate further the mechanism by which angiostatin inhibits MMP-2 expression during hypoxia, we measured p53 levels as this transcription factor is oxygen sensing (Vousden et al., 2007) and is one of a number of factors that regulate MMP-2 transcription (Bian et al., 1997; Lee et al. 2005). Under hypoxic conditions angiostatin (30 μg/ml) significantly decreased p53 expression by HMVEC-L compared to normoxic controls (0.3 ± 0.1 vs. 1.8 ± 0.2 arbitrary unit of density/mg protein; $P < 0.05$). (Figure 4B). Similarly, p53 knock down by endo-ribonuclease siRNA resulted in decreased MMP-2 production by the endothelial cells (Figure 4C). While pharmacological activation of p53 by a cell permeable activating peptide RI-TATp53C′-WT (activator II) or small molecule activator 2,5-bis-(5-Hydroxymethyl-2-thienyl)-furan (activator III) reversed the MMP-2 down-regulating effects of angiostatin during hypoxia (Figure 4D).

Control active p53 ELISAs confirmed the p53 activating nature of the two compounds (online supplementary Figure 2A).

3.3 Angiostatin Inhibits MMP-dependent Migration by Hypoxic Endothelial Cells

To test whether angiostatin inhibits MMP-dependent migration of hypoxic endothelial cells we established an *in vitro* migration assay in which endothelial cell migration is dependent on MMP digestion of gelatin (gelatinase activity). Our assay mimics the migration stage in which MMP-2 degrades the basement membrane type IV collagen that anchors endothelial cells within a blood vessel. Control experiments under normoxic conditions demonstrated that the broad-spectrum MMP inhibitor o-phenanthroline at a concentration known to inhibit MMP activity (1 mM) blocked HMVEC-L migration toward a VEGF gradient (online supplementary Figure 3). Further, knock down of MMP-2 using siRNA inhibited migration in our assay (Figure 5). Hence, we used this assay to assess the effects of angiostatin on MMP-dependent migration following exposure of endothelial cells to hypoxia.

Following hypoxic exposure, migration of angiostatin-treated (30 μg/ml) HMVEC-L was significantly impaired compared to corresponding hypoxic control HMVEC-L (11.6 ± 4.8 vs. 30.8 ± 9.7 cells per field of view; $P < 0.05$) (Figure 6A and B). Angiostatin-treatment during normoxia and hypoxia seemed to increase HMVEC -L migration over normoxic controls; however, this increase was not statistically significant (21.5 ± 7.6 vs. 27.6 ± 11.8 cells per field of view; $P > 0.05$).

3.4 Effects of Pathological Concentrations of Angiostatin on Endothelial Cell Apoptosis

Since hypoxic conditions may occur because of thrombus formation and since aggregating platelets release angiostatin from their intracellular stores, we investigated whether higher angiostatin concentrations would induce endothelial cell apoptosis. Upon aggregation with collagen platelets release up to 60 μg/ml of angiostatin, approximately doubling the levels that are normally seen in plasma of healthy individuals (56.7 ± 6.7 vs. 33.6 ± 9.2 μg/ml; $P < 0.05$) (Figure 7A and B). However, platelets may also release up to 200 pg/ml of VEGF (Figure 7C). Hence, we further investigated whether platelet-derived VEGF could counteract the anti-angiogenic effects of angiostatin. Under hypoxic conditions, at a concentration expected to be present following thrombus formation, 60 μg/ml angiostatin was able to overcome the protective effects of hypoxia and induced HMVEC-L apoptosis to the level of normoxic serum starved controls (39.4 ± 5.4% vs. 25.8 ± 7.9 % vs. 36.4 ± 8.4%; $P < 0.05$). Higher concentrations of VEGF (200 pg/ml) failed to prevent this angiostatin-induced apoptosis (36.4 ± 8.4% vs. 35.3 ± 6.7%, respectively; $P > 0.05$) (Figure 7D).

4. Discussion

The exact mechanisms by which angiostatin inhibits angiogenesis remain enigmatic in spite of a number of detailed studies (Claesson-Welsh et al., 1998; Gately et al., 1996; Griscelli et al., 1998; Hanford et al., 2003; Ji et al., 1998; Meena et al., 2004; Moser et al., 1999; Troyanovsky et al., 2001; Veitonmaki et al., 2004; Wahl et al., 2002a; Wahl et al., 2002b). A major reason why this likely remains the case is that the manner by which

angiostatin inhibits angiogenesis may change based on the microenvironment of a targeted endothelial cell (Wahl et al., 2005) and angiostatin concentration. Previously, it has been reported that a low pH_e microenvironment potentiates angiostatin's actions (Wahl et al., 2002a; Wahl et al., 2002b). A low pH_e can result from hypoxia, which can also stimulate angiogenesis (Keshet et al., 1992). Hence, an endothelial cell encountering angiostatin during hypoxia-stimulated angiogenesis is challenged with a dichotomy of signals. Therefore, the objective of our investigation was to study the effects of angiostatin on endothelial cells during hypoxia specifically focusing on their ability to survive and move.

We used angiostatin-isolated from human plasma since angiostatin is generated by circulating platelets and this form of the molecule would be most widely encountered by endothelial cells. We found that neither under normoxic nor hypoxic conditions did physiological concentrations of angiostatin (Jurasz et al., 2010) significantly induce the apoptosis of endothelial cells. This failure occurred in spite of a decrease in pH_e during hypoxia. In fact, hypoxia promoted endothelial survival consistent with our previous report that hypoxia prevents BNIP3-mediated apoptosis of endothelial cells (Jurasz et al., 2011). To confirm the functionality of the angiostatin used in our study we tested whether it would inhibit catalytic activity of an ectopic endothelial cell ATP synthase, a known angiostatin target. We found that human plasma-derived angiostatin inhibited extracellular ATP generation most potently under hypoxic conditions indicating that indeed it was active, and confirming that it like angiostatin K1-5 inhibits this ectopic ATP synthase (Veitonmaki et al., 2004). Although angiostatin was active during hypoxia, VEGF up-regulation protected endothelial cells from angiostatin-induced apoptosis and

likely from the intracellular acidosis induced during normoxia. We confirmed VEGF's protective effect by inhibiting its expression using siRNA. We found that when VEGF expression by hypoxic endothelial cells was inhibited angiostatin at a physiological concentration significantly induced endothelial cell apoptosis.

Since angiostatin inhibits angiogenesis under conditions in which hypoxia predominates, we explored whether angiostatin could inhibit angiogenesis via a mechanism alternate to apoptosis. We previously demonstrated that under low pH_e conditions angiostatin inhibits MMP-2 release from human umbilical vein endothelial cells (Jurasz et al., 2006). Hence, we investigated whether angiostatin inhibits expression of this protease during hypoxia. In-line with other studies, we found that hypoxia alone tended to increase MMP-2 mRNA levels compared to normoxic conditions (Ben-Yosef et al., 2002); however, this increase in MMP-2 mRNA did not translate into elevated amounts of MMP-2 protein. These results are consistent with the overall suppressive effects of hypoxia on protein translation (van den Beucken et al., 2006). Importantly, we found that angiostatin decreased MMP-2 mRNA in endothelial cells during hypoxia. This reduction in MMP-2 mRNA along with hypoxia's suppressive effects on translation resulted in a significant decrease in MMP-2 protein (72 and 64 KDa). The loss of the 64 KDa activated form of MMP-2 likely results due to both impaired expression of 72 KDa pro-MMP-2 and due to an impaired ability of MMP-14 (MT1-MMP) to activate the 72 KDa pro-form (Deryugina et al., 2001) since angiostatin also reduced HMVEC-L MT1-MMP during hypoxia. Numerous studies using MMP-2 and MMP-14 null mice have demonstrated the importance of these two MMPs to tumor, corneal, retinal, and chondral epiphysis angiogenesis and ischemia-induced

revascularization (Itoh et al., 1998; Fang et al., 2000; Takahashi et al., 2002; Kato et al., 2001; Zhou et al., 2000; Lee et al., 2005). Although MMP-2 and MMP-14 knockout mice are not embryonically lethal such as VEGF knockout mice (Carmeliet et al., 1996; Ferrara et al., 1996), both MMP-2 and MMP-14 null mice have significant growth impairment post-birth (Itoh et al., 1997; Zhou et al., 2000) suggestive of a defect in post-natal angiogenesis as seen in chondral epiphyses of MMP-14 null mice. Hence, MMP-2 and MMP-14 null mice have deficiencies in both pathological and physiological angiogenesis. One reason why the deficiencies in angiogenesis in mice with single MMP knockouts may not be as severe as seen in VEGF null mice may be due to the overlapping substrate specificity and compensatory effects of other MMPs. Indeed, the loss of both MMP-2 and MMP-9 has shown to lead to a dramatic reduction in tumor angiogenesis in double MMP-knockout mice (Masson et al., 2005). Since in our study angiostatin caused a decrease in the expression of both MMP-2 and MMP-14 during hypoxia, the inhibition in endothelial cell migration and consequently angiogenesis may be more pronounced than if only one MMP was affected.

We next examined the effects of hypoxia and angiostatin on the transcription factor p53, which regulates MMP-2 expression and is oxygen sensing (Bian et al., 1997; Lee et al., 2005; Vousden et al., 2007). We found that only under hypoxic conditions did angiostatin decrease p53 levels. P53 is basally regulated by ubiquitin-proteasome degradation system (Toledo et al., 2006), which is enhanced by acidosis (Mitch et al., 2004). Hence, one putative mechanism by which angiostatin may regulate p53 is by inducing an intracellular acidosis as a result of angiostatin inhibiting ATP synthase (Wahl et al., 2002a; Wahl et al., 2002b) thereby enhancing p53 degradation and

contributing to impaired MMP-2 expression. However, in our experimental system hypoxia protected endothelial cells from the angiostatin-induced intracellular acidosis which occurred only under normoxic conditions (Figure 1D). Alternatively and more likely, angiostatin may regulate p53 during hypoxia by potently reducing endothelial cell extracellular ATP production (Figure 2A). Extracellular ATP is important for binding to endothelial cell P2Y-purinoceptors and stimulating endothelial nitric oxide (NO) production (Kalinowski et al., 2003). NO is well known to increase expression/accumulation of p53 (Mebmer et al., 1994; Forrester et al., 1996), and previous studies have shown that angiostatin impedes endothelial NO production (Koshida et al., 2003; Takahashi et al., 2010). Interestingly, our preliminary data demonstrates that angiostatin reduces eNOS levels in hypoxic endothelial cells (online supplementary Figure 2B). Consequently, during hypoxia angiostatin may down-regulate p53 via reduced extracellular ATP-P2Y-purinoceptor-NO-signalling leading to a decrease in p53 binding to an adjacent enhancer site (RE-1) within the MMP-2 promoter (Lee et al., 2005).

We next investigated whether angiostatin would inhibit MMP-dependent migration of hypoxic endothelial cells. In our assay, we utilized gelatin, a MMP-2 preferred substrate, to mimic the type IV collagen basement membrane that normally anchors endothelial cells and is susceptible to MMP-2-dependent cleavage (Tryggvason et al., 1992). We found that angiostatin significantly impaired MMP-dependent migration of hypoxic, but not normoxic, endothelial cells through a gelatin-coated porous membrane. The ability of angiostatin to inhibit the expression of both MMP-2 and MT1-MMP by hypoxic endothelial cells suggests that angiostatin may impair multiple stages

of endothelial cell migration including MMP-2-dependent degradation of basement membrane type IV collagen and MT1-MMP-dependent degradation of type I collagen extracellular matrix (Sabeh et al., 2004).

Since platelets store and release angiostatin upon aggregation (Jurasz et al., 2003a), in instances when hypoxia results as a consequence of thrombosis, it is highly likely that angiostatin concentrations rise significantly above those found physiologically; thus, potentially changing the way in which angiostatin affects endothelial cells. To test this hypothesis we quantified angiostatin release and generation during platelet aggregation. Experiments carried out in human platelet rich plasma revealed that upon aggregation local plasma angiostatin concentration may double and rise to 60 μg/ml. Since aggregating platelets also release VEGF (Mohle et al., 1997), we also quantified its release and found that local plasma VEGF concentration may rise by 200 pg/ml in response to a strong platelet agonist. Hence, we next investigated the effects of higher angiostatin and VEGF concentration on endothelial cells during hypoxia. We found that at 60 μg/ml angiostatin-induced apoptosis of hypoxic endothelial cells, and a VEGF concentration that would likely be present in the microenvironment following thrombus formation failed to protect the cells from angiostatin-induced apoptosis.

Our findings have a number of important implications for angiogenesis regulation. First, in instances when inhibition of tumour angiogenesis is desirable, unless concentrations are sufficiently high, angiostatin will likely only prevent growth of new tumor vessels to hypoxic regions (likely in earlier disease stages) and not destroy vessels which are already established (advanced disease). This has important implications for angiostatin clinical trials, which may not have reached the optimal

biological dose of angiostatin nor have been tested within the optimal patient population (Beerepoot et al., 2003; Kurup et al., 2006). Alternatively, for angiostatin therapy to be successful at lower doses, it would likely need to be administered along with an agent that lowers VEGF or its signalling. Second, in instances where therapeutic angiogenesis is desirable it will likely be important to not only supply positive regulators of angiogenesis but also inhibitors of angiostatin (or its function) to remove the impediment on MMP-2 and MMP-14 expression, which would allow endothelial cells to move into hypoxic regions.

5. Conclusions

In summary, our findings have identified inhibition of MMP-2 and -14 expression and MMP-dependent migration as a novel mechanism by which angiostatin inhibits angiogenesis under pathological-like conditions. We believe this mechanism explains angiostatin's seemingly tumor angiogenesis-specific inhibitory effects and propose that it occurs under other pathological conditions in which hypoxia is present.

6. Acknowledgements

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Figure Legends

Figure 1. (A) Represenative flow cytometry dot plots and **(B)** summary data demonstrating the effects of angiostatin (30 μg/ml) on HMVEC-L apoptosis. N = 5. **(C and D)** Effects of hypoxia on HMVEC-L extracellular and intracellular pH, respectively. N = 3. *, $P < 0.05$ vs. Normoxia control. Angst – Angiostatin.

Figure 2. (A) Standard curve and summary data demonstrating angiostatin (30 μg/ml) mediated inhibition of extracellular ATP production by HMVEC-L. Sample luminescence was compared to luminescence generated by ATP standards. N = 5. *, $P < 0.05$ vs. normoxia control. **(B)** HMVEC-L VEGF production under normoxic (below detectable limits) and hypoxic conditions with and without angiostatin. N = 3. *, $P < 0.05$ vs. normoxia control. **(C)** Summary data (*left panel*) demonstrating VEGF silencing restores angiostatin-induced apoptosis of HMVEC-L under hypoxic conditions. Representative immunoblot (*right panel*) demonstrating siRNA-mediated knockdown of VEGF. Scram – scrambled. N = 4. *, $P < 0.05$ vs. normoxia control.

Figure 3. Angiostatin (30 μg/ml) inhibits HMVEC-L MMP-2 expression under hypoxic conditions. **(A)** Summary real-time PCR data. N = 4. *, $P < 0.05$ vs. hypoxia control. **(B)** Gelatin zymography (representative zymogram and summary data) and **(C)** MMP-2 immunoblot analysis (representative blot and summary data) of HMVEC-L lysates confirming angiostatin inhibits MMP-2 expression at the protein level under hypoxic but not normoxic conditions. N = 5. *, $P < 0.05$ vs. normoxic control.

Figure 4. (A) Representative immunoblot and summary data demonstrating angiostatin reduces HMVEC-L MMP-14 (MT1-MMP) during hypoxia (total – both pro- and active MT1-MMP). N = 3. *, P < 0.05 vs. normoxia control. **(B)** Representative immunoblot and summary data demonstrating decreased expression of the MMP-2 transcription factor p53 by angiostatin-treated HMVEC-L during hypoxia. N = 5. *, P < 0.05 vs. normoxia control. **(C)** Representative p53 and MMP-2 immunoblots (one of three) following esiRNA silencing of p53 under normoxic conditions. Scram – scrambled. **(D)** Representative gelatin zymography (one of two experiments) demonstrating that p53 activation reverses the inhibitory effects of angiostatin on MMP-2 expression by endothelial cells. Both p53 activator II (RI-TATp53C′-WT) and activator III (2,5-bis-(5-Hydroxymethyl-2-thienyl)-furan) were used at 3 μM.

Figure 5. (A) Representative microscopy and **(B)** summary data demonstrating MMP-2 dependence of HMVEC-L migration. Bars represent 100 μm. N = 3. *, P < 0.05 vs. Scrambled siRNA control. **(C)** Representative immunoblot confirming siRNA-mediated knockdown of MMP-2. Scram – scrambled.

Figure 6. (A) Representative microscopy and **(B)** summary data demonstrating that angiostatin (30 μg/ml) inhibits MMP-dependent HMVEC-L migration only under hypoxic conditions. N= 7. *, P < 0.05 vs. hypoxia control. Bars represent 100 μm.

Figure 7. (A) Representative human platelet aggregation traces. RP – resting platelets. Coll 10 – collagen 10 μg/ml. **(B)** Summary data quantifying platelet angiostatin and **(C)**

VEGF release in response to collagen. N=4. *, P < 0.05 vs. RP. **(D)** Summary data demonstrating that under hypoxic conditions higher concentrations of angiostatin (60 μg/ml) promote HMVEC-L apoptosis which is not rescued by the addition of recombinant (r) VEGF (200 pg/ml). N = 5. *, P < 0.05 vs. normoxia control.

Figure(s)

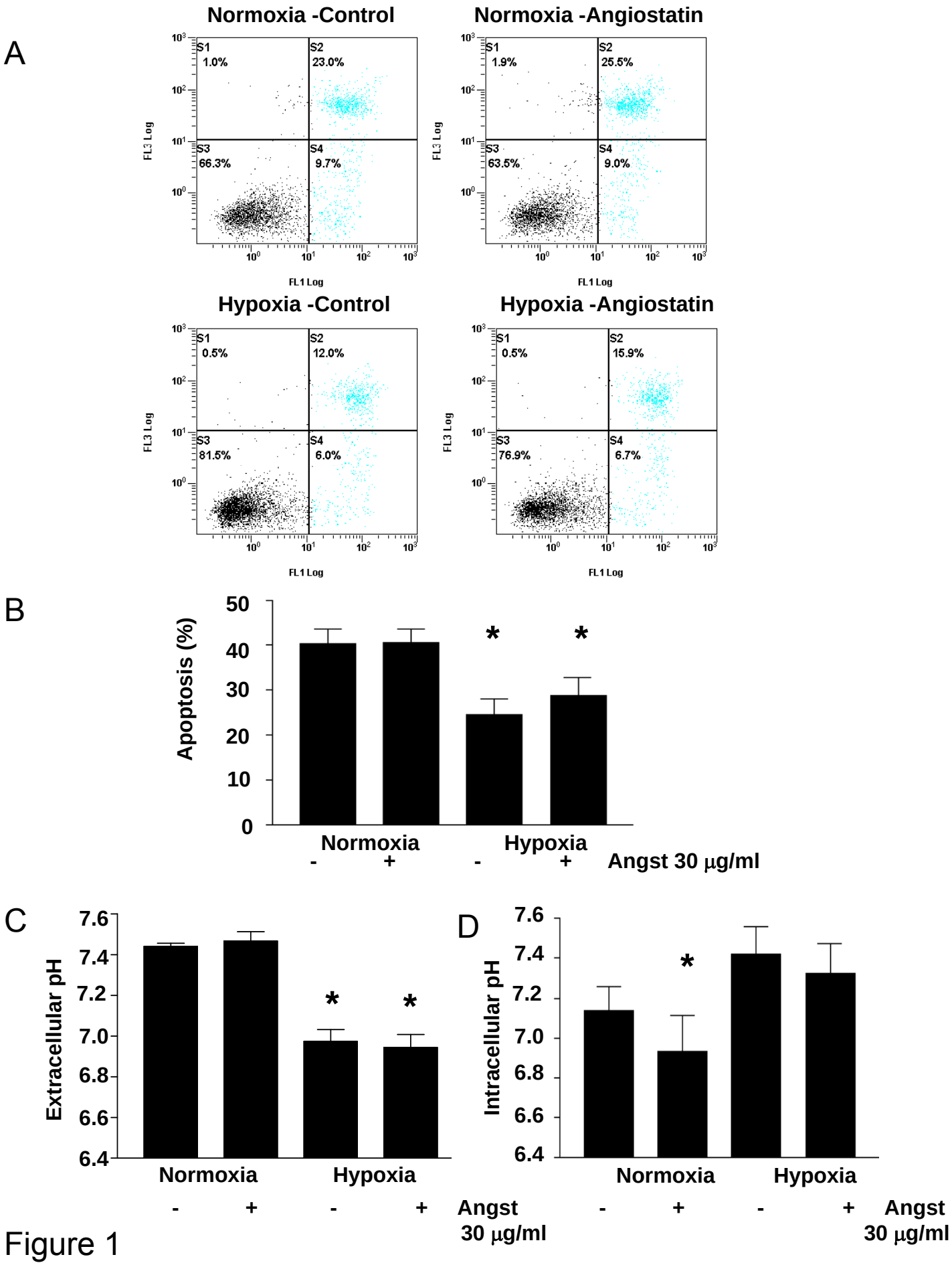

Figure 1

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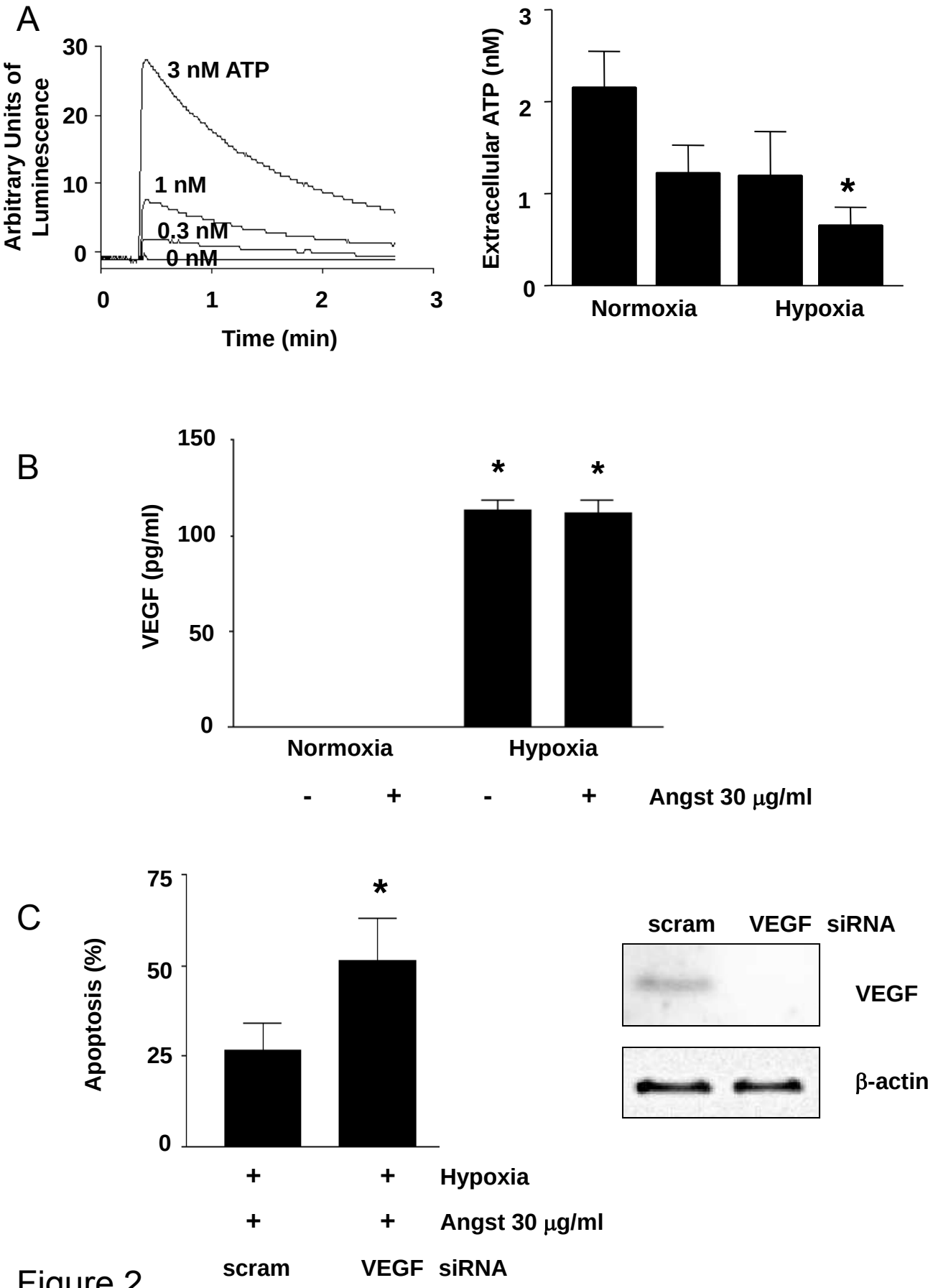

Figure 2

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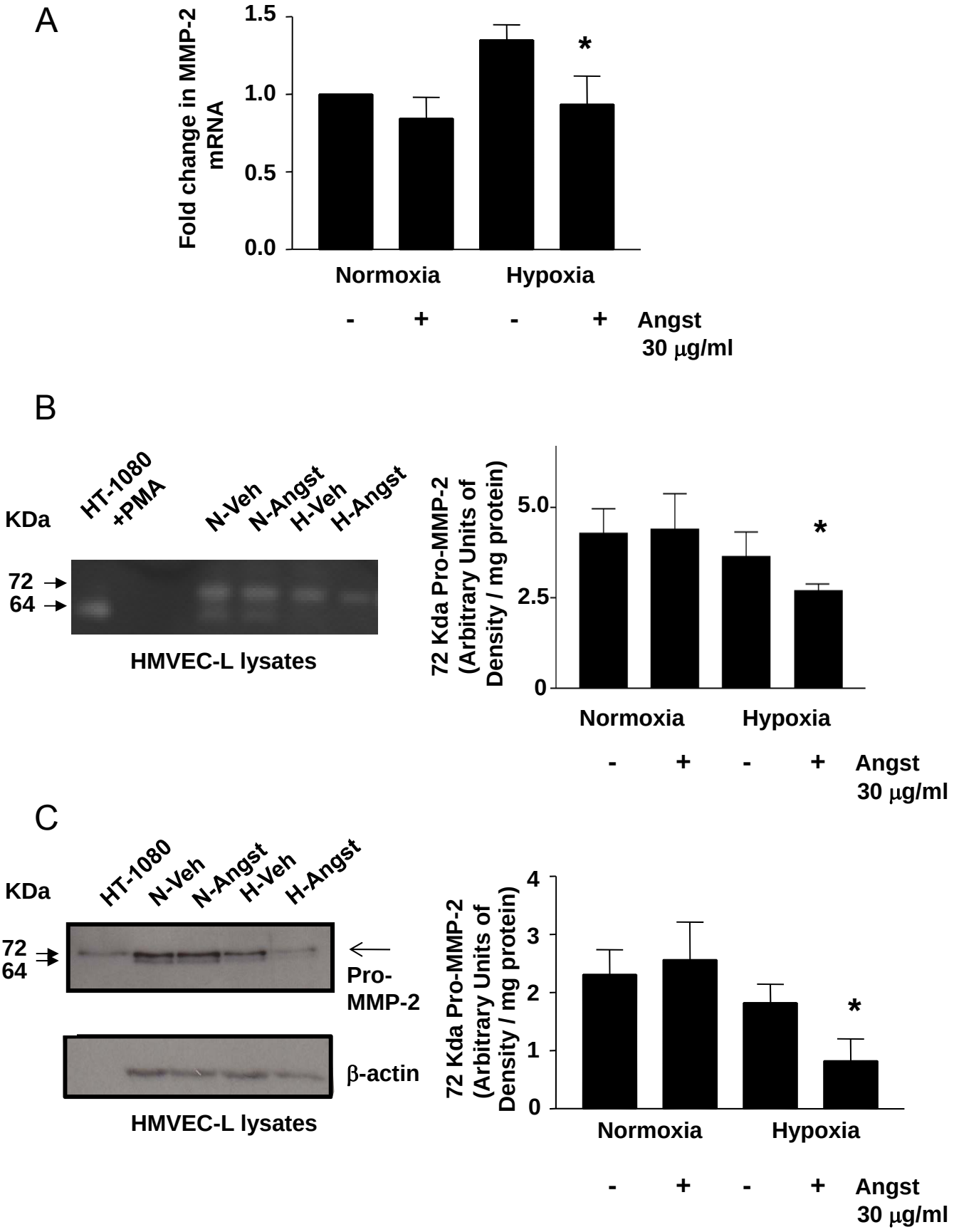

Figure 3

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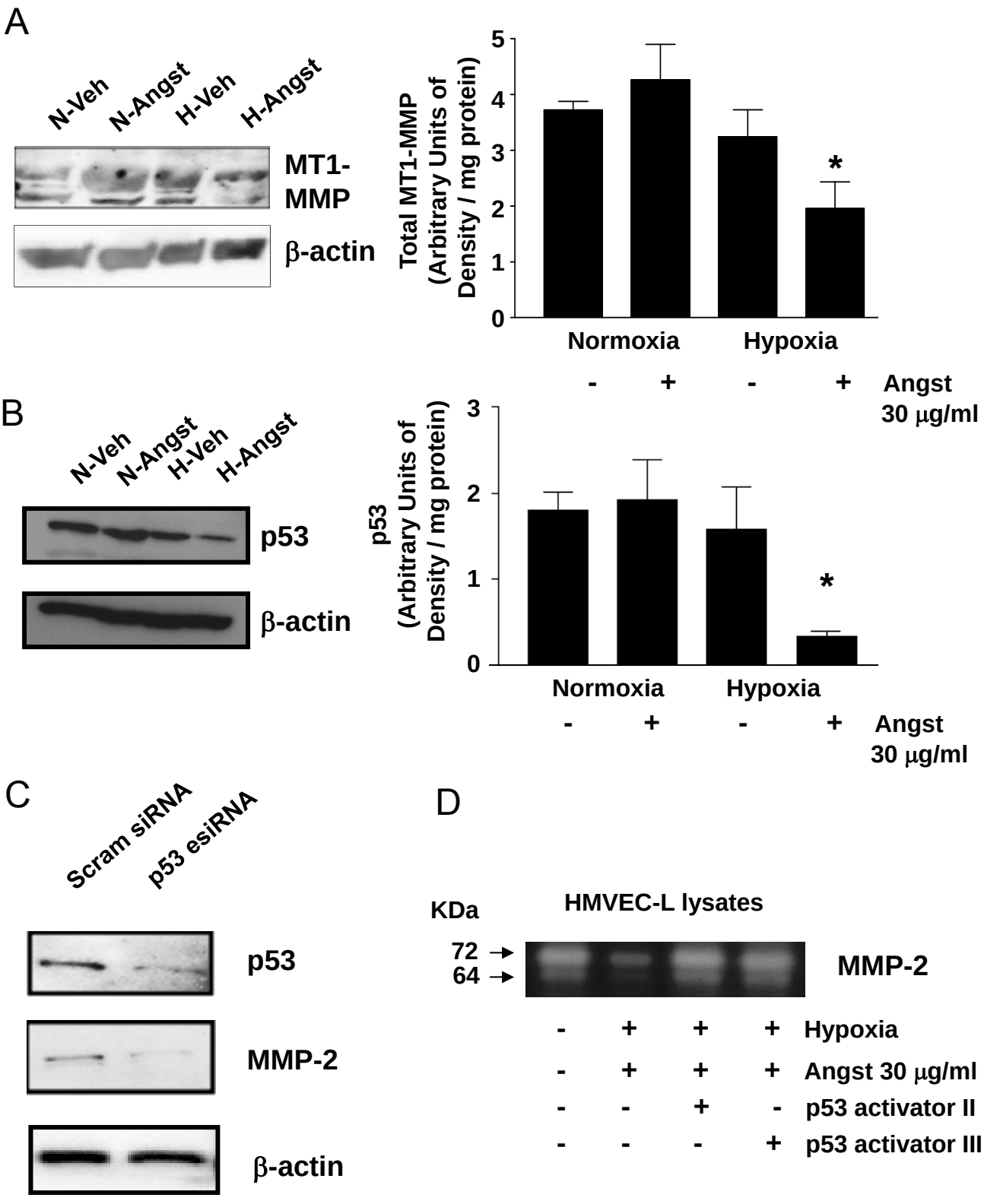
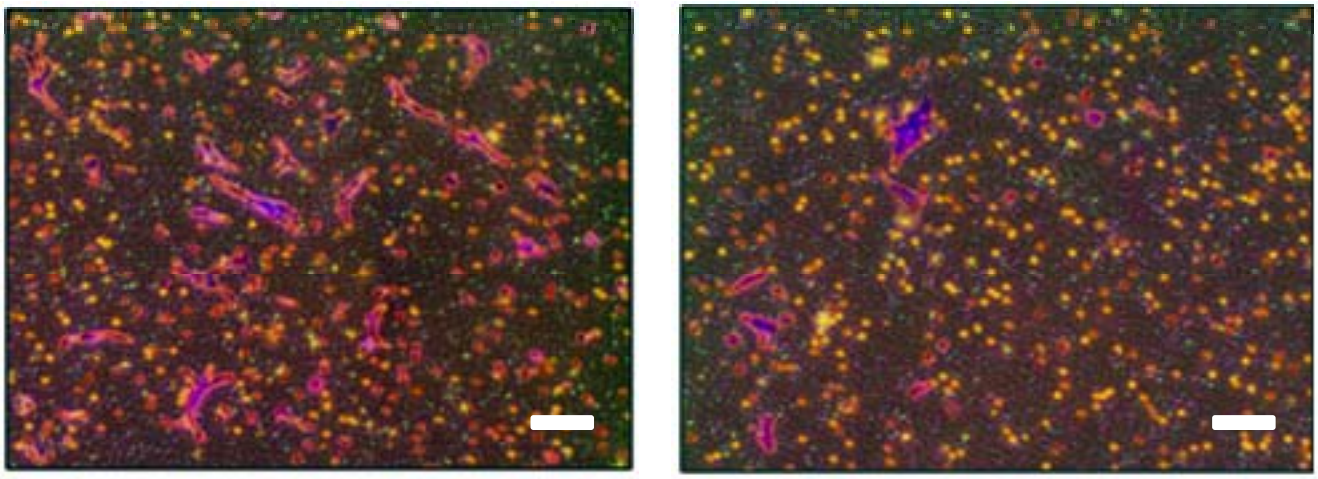


Figure 4

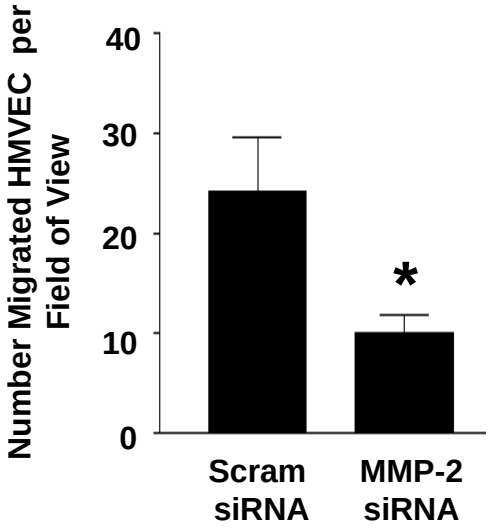
A



Scrambled siRNA control

MMP-2 siRNA 90 nM

B



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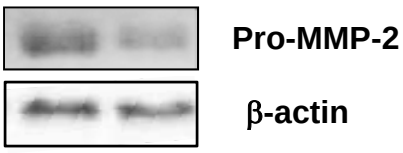

Figure 5

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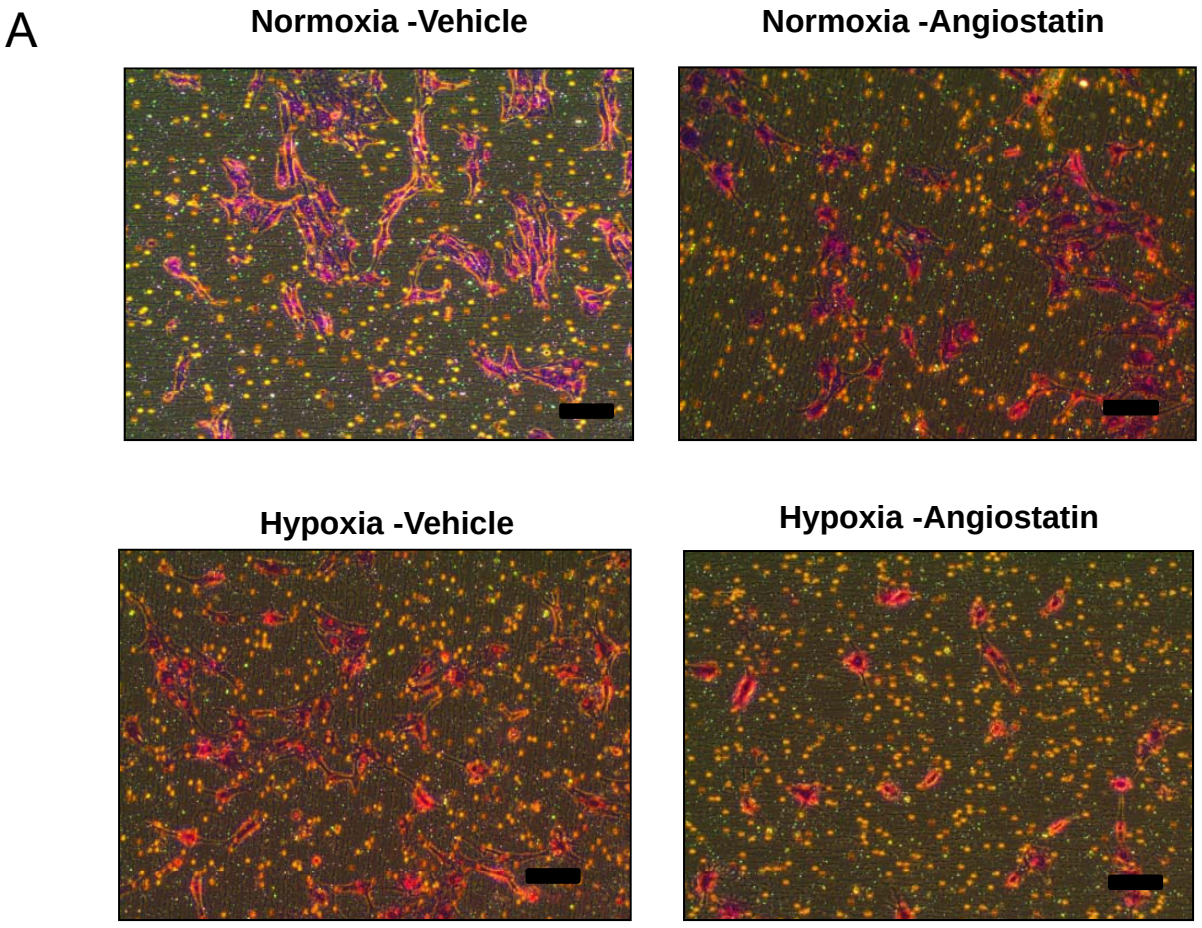

Figure 6

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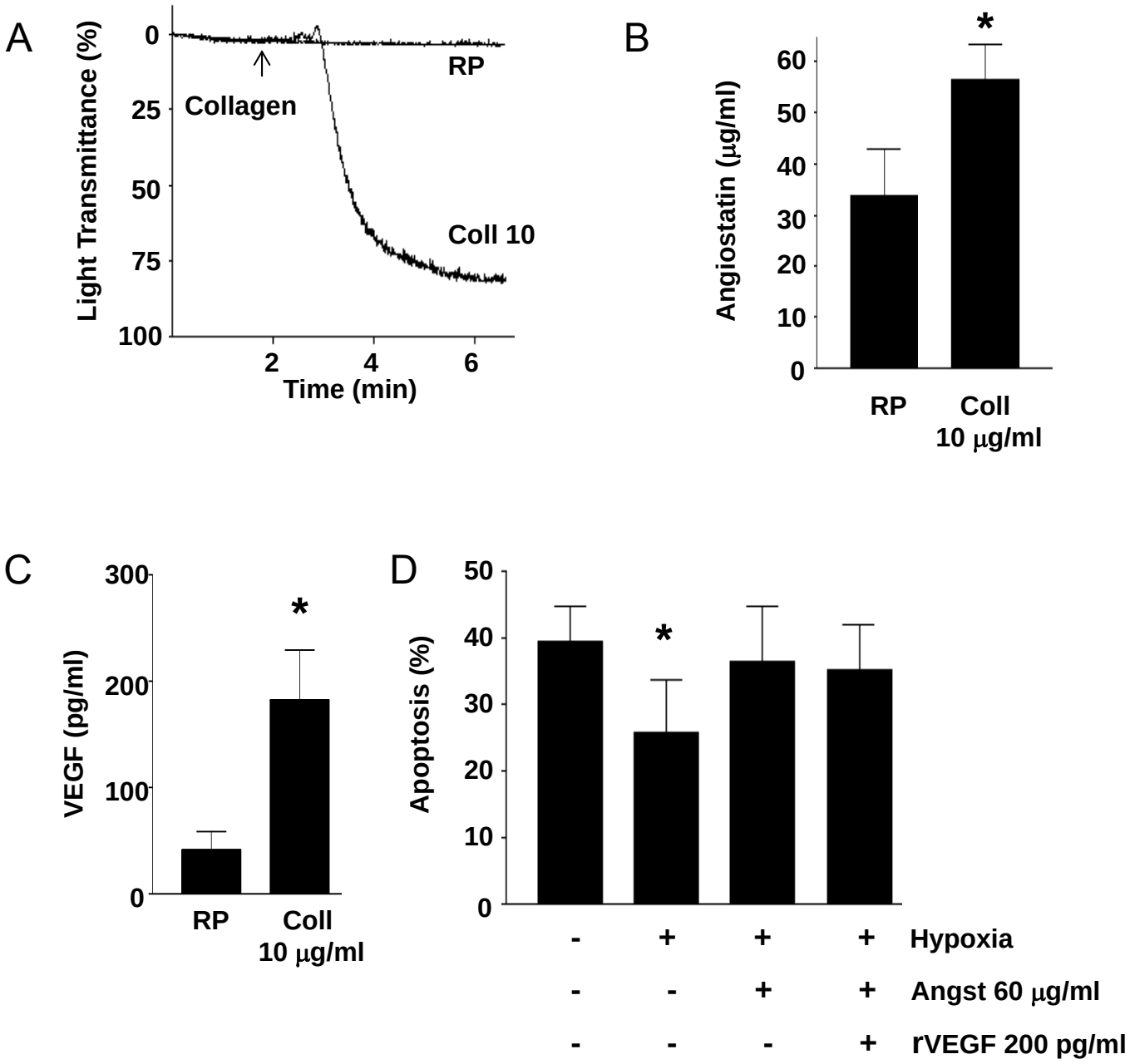
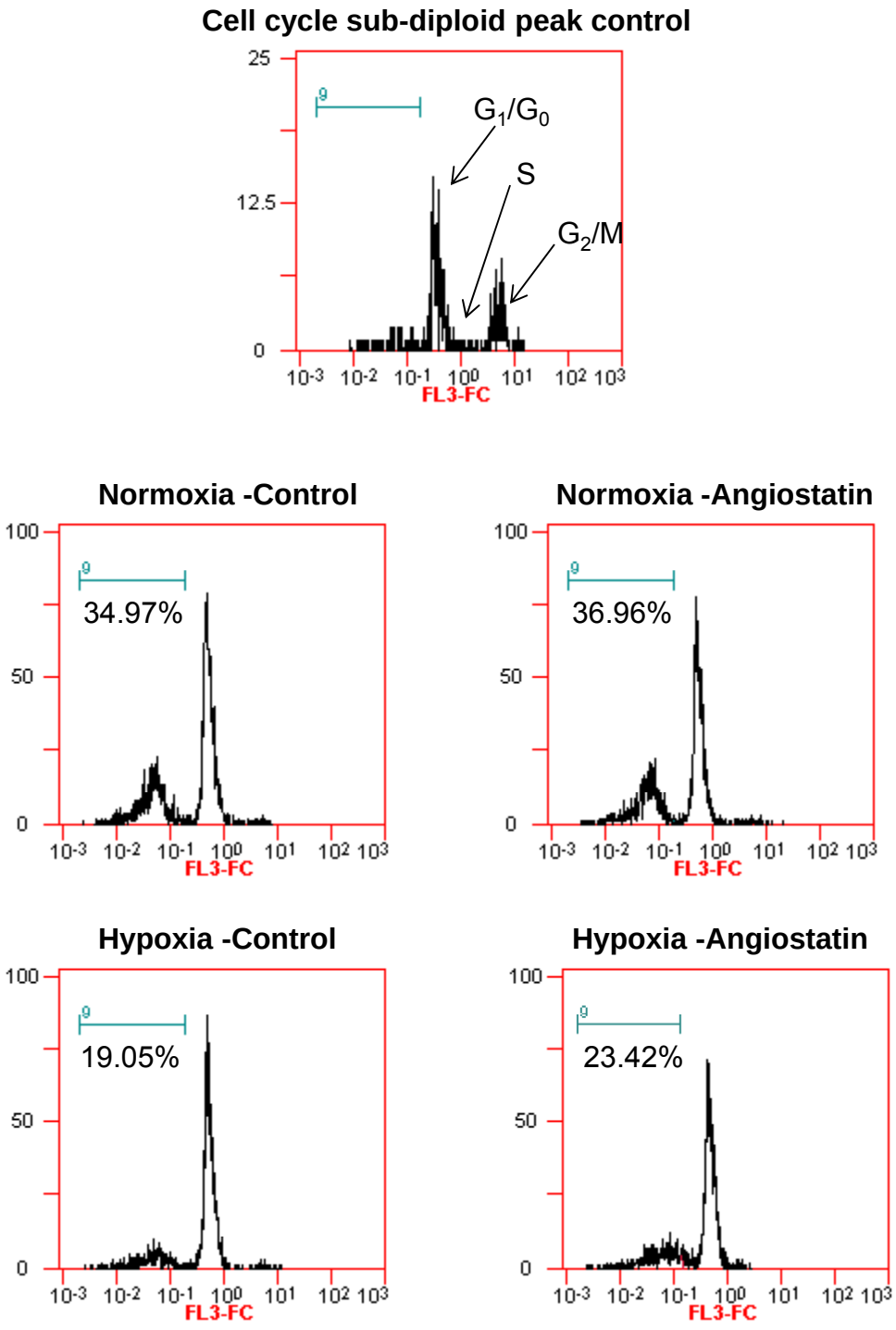
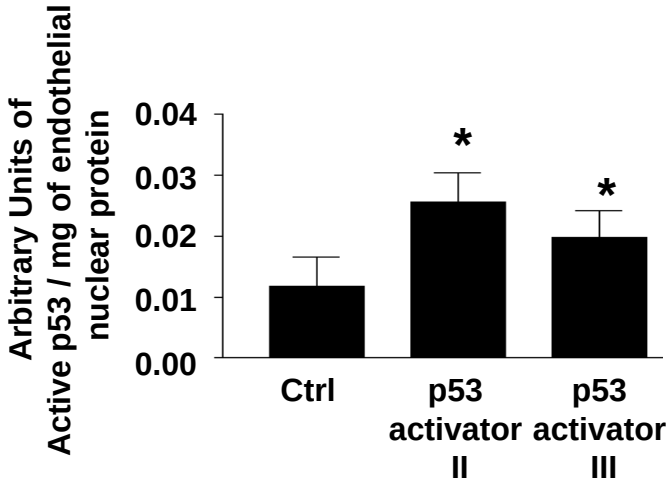


Figure 7



Supplemental figure 1. Sub-diploid peak flow cytometry analysis of HMVEC-L apoptosis following treatment with 30 μg/ml angiostatin. Region 9 represents the sub-diploid peak.

A



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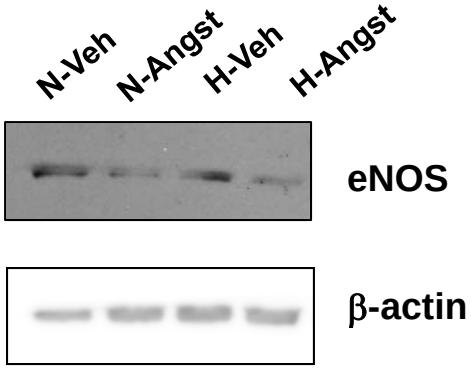
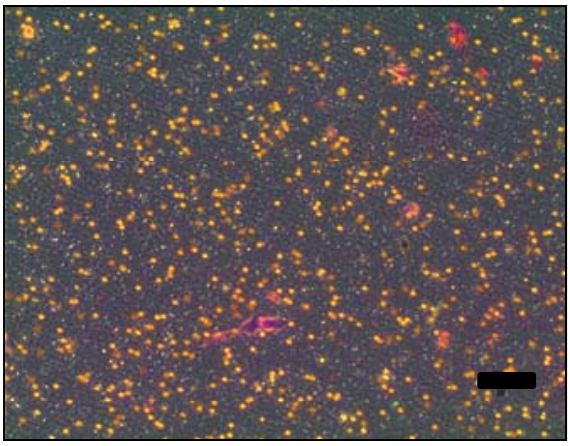
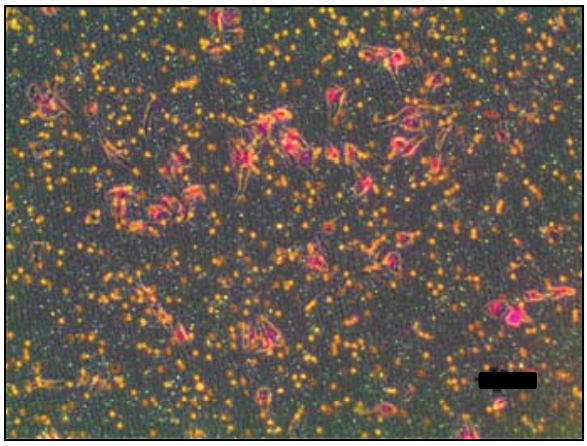


Supplemental figure 2. (A) Summary active p53 ELISA data confirming the p53 activating nature of RI-TATp53C′-WT (activator II) (3 μM) and 2,5-bis-(5-Hydroxymethyl-2-thienyl)-furan (activator III) (3 μM) in HMVEC-L. N = 4. *, P < 0.05 vs. control. **(B)** A single immunoblot demonstrating the eNOS down-regulating effects of angiostatin (30 μg/ml) within HMVEC-L during hypoxia. N – normoxia, H – hypoxia, Veh – vehicle, Angst – angiostatin.

A

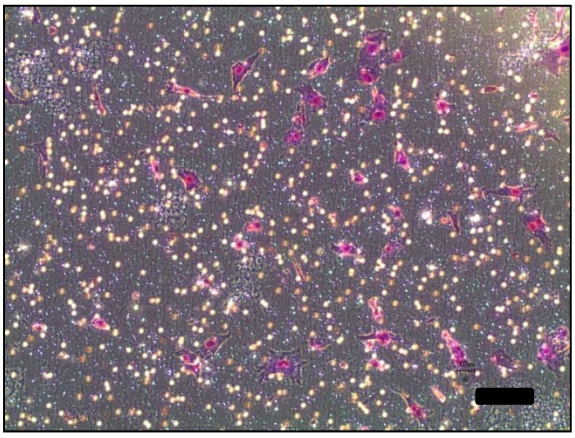


Control

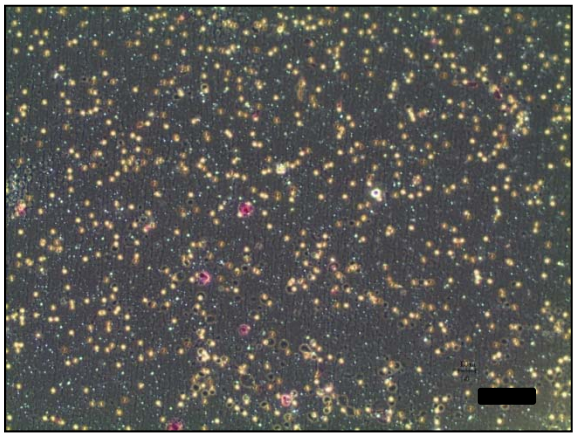


VEGF (5 ng/ml)

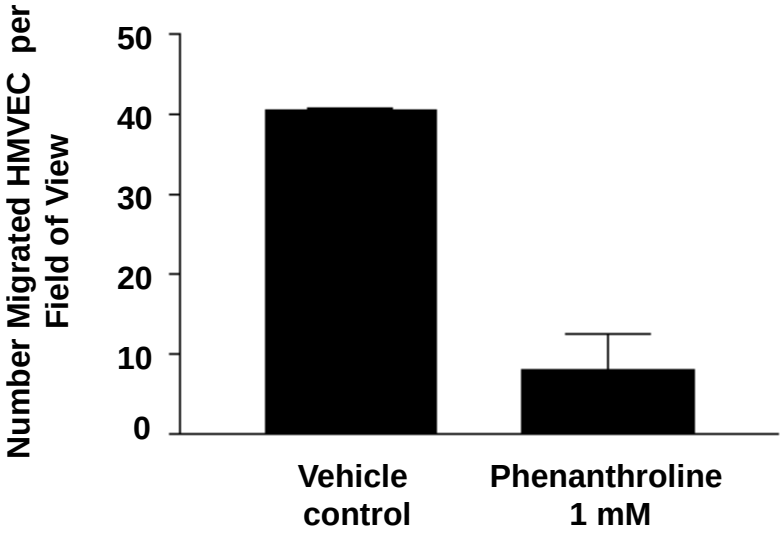
B



VEGF (5 ng/ml) + Vehicle control



VEGF (5 ng/ml) + Phenanthroline 1 mM



Supplemental figure 3. (A) Representative microscopy of negative and positive control migration assays. **(B)** Representative microscopy and summary data demonstrating MMP-dependence of HMVEC-L migration. Bars represent 100 μm. N = 2.