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Suppression of interactions between prostate tumor cell integrin $\alpha_v\beta_3$ and endothelial ICAM-1 by simvastatin inhibits prostate cancer micrometastasis

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Abstract

Cancer micrometastasis relies on the ability of cancer cells to secrete angiogenic modulators, to interact with the vascular endothelium, and to overcome the resistance offered by the endothelial-barrier. Being an essential step prior to metastasis, blockage of micrometastasis can have potential applications in cancer therapy and metastasis prevention. Due to poorly known molecular mechanisms leading to micrometastasis, developing therapeutic strategies to target prostate cancer utilizing drugs that block micrometastasis is far from reality. Here we demonstrate the potential benefits of simvastatin in the inhibition of prostate cancer micrometastasis and reveal the novel molecular mechanisms underlying this process. First, we showed that simvastatin inhibited the ability of human PC3 prostate cancer cells for transendothelial migration *in vitro*. Second, our data indicated that simvastatin modulates the expression of tumor derived factors such as angiopoietins and VEGF-A at the mRNA and protein levels by the PC3 cells, thus preventing endothelial-barrier disruption. Third, simvastatin directly activated endothelial cells and enhances endothelial-barrier resistance. Apart from this, our study revealed that simvastatin-mediated effect on PC3 micrometastasis was mediated through inhibition of integrin $\alpha_v\beta_3$ activity and suppression of interaction between prostate cancer cell integrin $\alpha_v\beta_3$ with endothelial ICAM-1.

Keywords

Prostate cancer; micrometastasis; integrin $\alpha_v\beta_3$; ICAM-1; simvastatin

Introduction

Although slow growing, prostate cancer is the leading cause of cancer-related death among men. Using the prostate specific antigen (PSA) testing and Gleason score analysis (USPSTF,

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Conflict of interest
None

2012), although many patients get diagnosed for prostate cancer at an early stage, many of them later develop castration-resistant metastatic prostate cancer. This demands additional research in developing procedures for prevention and/or the treatment of advanced stage prostate cancer.

Statins are lipid-lowering agents inhibiting HMG-CoA reductase enzyme, a key component of the cholesterol synthesis machinery (Alberts et al., 1980). Apart from this, statins elicit pleiotropic effects on multiple cell types in regulating various cellular functions (Liao and Laufs, 2005). Previous studies have shown that while statins are vascular protective via activation of endothelial cells (Li and Losordo, 2007), it inhibits proliferation of malignant cells such as hyper-active smooth muscle cells in the atherosclerotic plaques, leading to plaque stabilization and decreasing coronary artery disease-related mortality (Kwak et al., 2003, Takemoto and Liao, 2001). This clearly indicates the 'normalizing' potential of statins by activating cells at rest and inhibiting hyper-active malignant cells. This normalizing ability of statins has gathered specific interests for its potential applications in cancer for prevention and chemotherapy in combination with other drugs, thereby cancer therapy can be more effective and at the same time side-effects of chemotherapy can be minimized. In support of this view, a recent report indicates that long-term use of statins help to reduce cancer-related mortality by 15%, equivalent to the success rate of chemotherapy (Nielsen et al., 2012).

During the last several years, various statins have been tested for their anti-cancer efficacy on different cancer cell types *in vitro* and animal models *in vivo* (Jakobisiak and Golab, 2010). Despite several controversies on the beneficial vs. adverse effects of statins on various cancers, investigations validating the use of statins for prostate cancer therapy have been highly promising (Papadopoulos et al., 2011). A recent clinical study has reported 45% reduction in the biochemical recurrence of prostate cancer after radical prostatectomy in patients taking statins (Hamilton et al., 2010). Statins have been reported to be safe for humans even at doses 10–50 times higher than that is prescribed for cardiovascular disease (Holstein et al., 2006, Gauthaman et al., 2009). Previous studies from our group has demonstrated the anti-cancer efficacy of simvastatin, a highly lipophilic statin on androgen-responsive LNCaP cells and androgen-insensitive PC3 prostate cancer cell lines *in vitro* and tumor xenografts *in vivo* (Kochuparambil et al., 2011). Simvastatin also induced apoptosis in prostate cancer cells via simultaneous modulation of intrinsic cell survival and extrinsic apoptotic pathways (Goc et al., 2012b). Simvastatin-induced effects on prostate cancer cells were mainly mediated through the inhibition of Akt, a serine-threonine kinase that has been implicated to be essential for prostate cancer progression and metastasis (Hammarsten et al., 2012, Goc et al., 2011, Goc et al., 2012d). Our studies have also demonstrated the pivotal role of Akt in mediating prostate cancer micrometastasis via activation of integrin $\alpha_v\beta_3$ (Goc et al., 2012d), which have been reported to be elevated in prostate cancer cells (McCabe et al., 2007).

The process of micrometastasis involves intravasation and extravasation of cancer cells into the blood vessels and is a pre-requisite for the metastasis of prostate cancer cells to distant tissues such as bone and lungs (Tantivejkul et al., 2004). Due to this rate-limiting nature of the micrometastasis step in cancer progression, its blockage can be developed into an

effective strategy for the prevention of prostate cancer metastasis, thus providing longer window for the surgical removal of the cancer tissue. Since simvastatin inhibits Akt pathway in prostate cancer cells (Kochuparambil et al., 2011) and Akt is important for prostate cancer micrometastasis (Goc et al., 2012d) and vascular maturation (Chen et al., 2005, Somanath et al., 2008), this combined with the vascular protective role of statins lead us to hypothesize that simvastatin can be highly effective in preventing prostate cancer micrometastasis.

In the current study, we explored the effects of simvastatin on prostate cancer micrometastasis. We first demonstrated that simvastatin inhibited expression of VEGF and enhanced expression of angiopoietin-1 at the RNA and protein levels as well as other signaling molecules such as IGF-I, integrins and PDGFβ etc., implicating its effects on stabilizing the endothelial-barrier. Our results provide strong evidence that while simvastatin performs vascular normalization through Akt-mediated activation of endothelial cells, thus protecting the endothelial-barrier; it prevents micrometastasis of prostate cancer cells *in vitro* via suppression of interactions between prostate cancer cell integrin $\alpha_v\beta_3$ and endothelial ICAM-1. To our knowledge, we provide the first evidence demonstrating the potential application of statins in the prevention of interactions between prostate cancer and the endothelium and inhibition of prostate cancer micrometastasis.

Materials and Methods

Cell culture

PC3 human prostate cancer cells were grown in DMEM/High glucose media supplemented with 10% FBS and 100 U/mL of penicillin-streptomycin (Fisher Scientific, Pittsburgh, PA). Human Microvascular Endothelial Cells (HMVECs) were grown in EBM-2 Basal Medium supplemented with EGM-2 MV SingleQuot Kit and Blasticidine (12.5 mg/ml) (Lonza, Fisher Scientific, Pittsburgh, PA).

Real-time PCR

Upon reaching 90% confluence, cells were treated with activated Simvastatin 25 μM vs. control for 12 h. Cells were harvested and lysed for mRNA using RNeasy Mini Kit (Qiagen, Valecia, CA), cDNA was then produced from mRNA using RT^2 First Strand Kit (SA Biosciences, Valecia, CA). A total of 25 μg of cDNA was applied on each Cancer PathwayFinder PCR Array® (SA Biosciences, Valecia, CA) well, and PCR was run using an Eppendorff realplex2 equipment. Results were plugged into the associated tool available in SA Biosciences website to compare difference in expression using ΔΔC method after normalization to housekeeping genes.

Protein precipitation and immunoblotting

A ratio of 1:4 Trichloroacetic acid and the conditioned media was mixed and kept overnight in 4°C. Precipitated proteins were pelleted at 14000 rpm, washed twice with acetone and dried for 5 minutes at 95°C and then mixed with lamelli sample buffer and β-mercaptoethanol (9:1) followed by boiling for 5 min at 95°C. Equal volumes of samples were loaded in a 12% SDS-PAGE and subjected for Western blotting as done previously (Goc et al., 2012a).

Western blotting

HMVECs were treated with Simvastatin vs. control for 0, 1, 6 and 12 h and lysates were prepared using RIPA buffer as previously described (Kochuparambil et al., 2011). Equal amounts of protein (50 μg) were loaded on SDS-PAGE and subjected for Western analysis using specific antibodies for pS473Akt, pS9,21GSK, VE-Cadherin, β-Catenin (All from Cell Signaling, Danvers, MA), and Actin (Sigma Aldrich, St. Louis, MO).

Immunocytochemistry

PC3 cells were plated on glass cover-slips, grown to confluence, treated with simvastatin vs control. At endpoint, media was removed, cells were washed using PBS and fixed using 4% paraformaldehyde washed thrice with PBS, blocked using 5% goat serum albumin/0.3% Triton X-100 for 30 min, incubated with primary antibodies for VE-Cadherin, or β-Catenin (both from Cell Signaling, Danvers, MA) overnight at 4°C. Cells were washed three times with PBS and then incubated with secondary antibodies labeled with alexa-488 (goat anti-mouse) or alexa-597 (goat anti-rabbit) for 1 h followed by three times washing with PBS. Cover-slips were mounted on to the microscope slides using DAPI-containing vectashield (Vector laboratories, Burlingame, CA). Slides were viewed using Carl-Zeiss Fluorescent microscope (Carl Zeiss, Germany).

Electric Cell Substrate Impedance Sensing (ECIS)

Electric Cell Substrate Impedance Sensing (ECIS) (Applied Biophysics, Troy, NY) was performed according to manufacturer's recommendations. Briefly, arrays were first washed with 10 mM glycine for 10 minutes, washed with media, stabilized using the device, and HMVECs were plated on the array wells. Upon reaching monolayer, cells were treated with: Simvastatin (5, 10 and 25 μM vs. control), detached PC-3 cells, or media collected from Simvastatin-treated PC-3 cells. For cell detachment, cells were first treated with Simvastatin (5, 10 and 25 μM vs. control), media was washed with PBS, and then a sterile Ethylenediamine Tetraacetic acid (EDTA, 20 mM) containing PBS was added to cells (for 5–7 minutes, 37°C) to detach them from ECM without digesting cell surface receptors. Cells were then collected, pelleted, re-suspended in media and counted. A total of 5×10^4 cells were added to each ECIS array well. For media collection, cells were treated with Simvastatin for 3 h and then media was removed, washed with serum free medium and incubated further in serum free medium to collect secreted factors over 12 h. Following this, media was collected, centrifuged to remove the cell debris, and a total of 200 μl of the conditioned media was added to each ECIS array well. For AP7.4 (β3-integrin activation antibodies; kindly provided by Thomas Kunicki) experiments, PC-3 cells were grown to a 70% confluence, treated with Simvastatin for 10 h, treated with AP7.4 (5 μg/ml) for another two hours, collected using cell dissociation buffer followed by adding to the ECIS array wells containing endothelial monolayer. Real-Time resistance/impedance was measured and recorded by the equipment automatically.

Cancer cell endothelium adhesion assay

PC3 cells were infected with GFP-expressing empty vector adenovirus particles. Briefly, 10 μl of a 10^9 PFU of adenovirus particles were added to a 6-well plate. 48 h later, cells were

treated with Simvastatin (25 μM) vs. control for 4 h and 16 h, followed by plating of cells on top of a monolayer of HMVECs for adhesion. After 1 h, excess cells that were not attached were washed off, fixed with 2% paraformaldehyde and viewed under fluorescent microscope. Attached cells were identified by the GFP fluorescence and quantified.

Integrin $\alpha_v\beta_3$-ligand binding assays

PC3 cells were plated in a 12-well plate and cultured until monolayer, then treated with simvastatin vs. control for 12 h. Media was removed and integrin $\alpha_v\beta_3$ specific ligand Fibrinogen-Alexa-488 (12.5 μg/ml or 100 μg/ml, respectively; Invitrogen, Calrsbad, CA) was added to the wells for 40 min. Excess Fibrinogen-Alexa-488 was washed off and fluorescence was measured at 488/519 using a BioTek multi-plate reader (Biotek, Winooski, VT).

Activation-dependent ligand, WOW-1 Fab (30 μg/ml; kindly gifted by Sanford Shattil, Scripps Research Institute, CA), was used to determine the activation status of integrin $\alpha_v\beta_3$ in PC-3 cells as previously described (Goc et al., 2012d). A monolayer of PC3 cells was treated with simvastatin for 12 h. WOW-1 Fab (30 μg/ml) was added and incubated for additional 40 min. Wells were washed once with PBS, incubated for 1 h with secondary goat anti-mouse antibody labeled with Alexa-488, washed three times with PBS, fixed with 2% paraformaldehyde and fluorescence was measured at 488/519 using a BioTek multi-plate reader (Biotek, Winooski, VT).

PC3 cell ICAM-1 adhesion assay

Human soluble ICAM-1 (R&D Systems, Minneapolis, MN) at a final concentration of 12.5 μg/ml was plated on to each well of a 12 well plate and PC3 cells, pre-treated with various concentrations of simvastatin (5 and 25 μM) were added on top of the wells pre-coated with ICAM-1 at a concentration of 1×10^4 cells/well. After 40 Min, wells were washed three times with PBS to remove unbound cells and adhered cells were stained with crystal violet and counted.

Assessment of tumor angiogenesis in nude mice tumor xenografts

Tumor xenografts were implanted and collected as done previously (Kochuparambil et al., 2011). Tumor sections were stained for antibodies specific to laminin (Sigma, St. Louis, MO) followed by incubation with Alexa-488 labeled secondary antibodies (Invitrogen, Calrsbad, CA). Slides were then mounted on slides using vectashield with DAPI and imaged using Carl-Zeiss Fluorescent microscope (Carl Zeiss, Germany).

Statistical analysis

All the data are presented as mean ± SD. To determine significant differences between treatment and control values, we used the Student's two-tailed t test. The significance was set at 0.05 levels (marked with symbols wherever data are statistically significant).

Results

Simvastatin inhibits PC3 cell interactions with the endothelium

Although the effects of simvastatin-modulated factors on prostate cancer cells have been demonstrated (Goc et al., 2012c, Brown et al., 2012, Vainio et al., 2011) our results suggested that the simvastatin-mediated inhibition of prostate cancer cell micrometastasis is reliant on tumor-host (or EC) cell interactions. The first line of evidence was the modulation of integrin expression in PC3 cells by simvastatin (Table 1). Hence, in the next step, we investigated the effect of simvastatin on interactions between prostate tumor cell and the endothelial cells. To do this, equal number of washed GFP transfected PC3 cells treated with simvastatin for 12 h were plated onto a monolayer of endothelial cells for a 1 hour adhesion assay. Our results indicated that simvastatin significantly inhibited adhesion of PC3 cells to the endothelial monolayer in a time-dependent manner (Figure 1 A–D). While 20% decrease in PC3 cell adhesion to the endothelium was observed in 4 h post simvastatin treatment (Figure 1 A and B), there was >60% inhibition of PC3 cell adhesion to the endothelium after 16 h (Figure 1 C and D).

Simvastatin inhibits expression of prostate tumor cell-derived factors

Next, we sought to identify the key growth factors and cytokines regulating simvastatin-mediated inhibition of HMVEC-barrier disruption by PC3 tumor cell-derived factors. First, we performed a qRealTime-PCR array for PC3 cells pre-treated with 25 μM simvastatin for 12 h. Our results indicated that while simvastatin inhibited the mRNA expression of pro-angiogenic and/or endothelial-barrier disrupting growth factors such as VEGF-A, IGF-1 and angiopoietin-2, it resulted in the increased expression of angiopoietin-1, a growth factor known to arrest endothelial-barrier break-down and enhance vascular maturation (Figure 2A and Table 1). These results were corroborated with our Western analysis data, which showed that while simvastatin treatment resulted in significant decrease in the secretion of VEGF-A (2-fold) into the conditioned media by the PC3 cells, secretion of angiopoietin-1 was significantly increased (2-fold) (Figure 2B–D). These results demonstrated the effect of simvastatin on the secretion of various pro- and anti-angiogenic factors such as VEGF-A and angiopoietins in the modulation of endothelial-barrier resistance.

Simvastatin has no significant effect on the rate of tumor angiogenesis

Since expression of pro-angiogenic and vascular permeability modulators VEGF-A and Angiopoietin-1 in PC3 cells were altered by simvastatin treatment, we investigated the effects of simvastatin on tumor angiogenesis *in vivo* in PC3 tumor xenografts developed in athymic nude mice (Kochuparambil et al., 2011) (Figure 3). Interestingly, simvastatin treatment did not inflict any significant changes in the vascular area in PC3 cell tumor xenografts as evidenced by the laminin statining, demonstrating that simvastatin is more involved in the normalization of tumor vasculature than modulating tumor angiogenesis.

Inhibition of prostate cancer cell interactions with the endothelium is mediated through integrin $\alpha_v\beta_3$

Cell-surface integrin $\alpha_v\beta_3$ has been implicated in mediating interactions between lymphocytes and vascular endothelial cells in mediating diapedesis (Steffen et al., 1994). Integrin $\alpha_v\beta_3$ is highly expressed in invasive and metastatic prostate cancer cells and our previous study has demonstrated the role of Akt-integrin $\alpha_v\beta_3$ cooperation in mediating prostate cancer cell micrometastasis (Goc et al., 2012d). Therefore, we tested if simvastatin-mediated inhibitory effects on prostate cancer cell interactions with the endothelium involved integrin $\alpha_v\beta_3$. Our study utilizing Alexa 488-labelled fibrinogen, a specific ligand for integrin $\alpha_v\beta_3$ and WOW-1 Fab that detects only active integrin $\alpha_v\beta_3$ on the cell surface demonstrated significantly impaired interaction of fibrinogen-Alexa 488 (Figure 4A) and WOW-1 (Figure 4B) with PC3 cells pre-treated with different doses of simvastatin for 12 h, indicating impaired affinity of integrin $\alpha_v\beta_3$ for its extracellular matrix ligands.

To confirm this further, we performed a rescue experiment utilizing AP7.4, a specific clone of antibodies that bind to the extracellular domain of the cell-surface integrin $\alpha_v\beta_3$ and induce activating conformational changes. Treatment of PC3 cells with AP7.4 enhanced their micrometastasis, compared to the control (Figure 4C). While simvastatin impaired the ability of PC3 cells to micrometastasize, our results indicated that pre-treatment of PC3 cells with AP7.4 significantly rescued simvastatin-mediated inhibition of micrometastasis *in vitro* on HMVECs (Figure 4C and D).

Simvastatin suppresses interactions between PC3 cell integrin $\alpha_v\beta_3$ and endothelial ICAM-1

One mechanism by which circulating cells adhere to endothelium to mediate diapedesis is by utilizing their surface integrin $\alpha_v\beta_3$ to bind to specific ligands-cum-adhesion molecules on the endothelium such as VCAM-1 and ICAM-1 (Steiner et al., 2010). Since integrin $\alpha_v\beta_3$ is also necessary for prostate cancer micrometastasis, we tested whether simvastatin treatment had any effect on interactions between PC3 cell-surface integrin $\alpha_v\beta_3$ and ICAM-1, adhesion molecules that are abundant on endothelial cell surface. Our data indicated that treatment with various doses of simvastatin significantly inhibited PC3 cell interaction with ICAM-1 (Figure 5A). In order to confirm that observed inhibition of interactions between PC3 cell-surface integrin $\alpha_v\beta_3$ and ICAM-1 due to simvastatin treatment is not specific to only one cell type, we performed adhesion assay using a second human invasive prostate cancer LNCaP-C42 cell line to study the effect of simvastatin on culture plates pre-coated with fibrinogen and ICAM-1. Treatment with 25 μM simvastatin resulted in significant inhibition of LNCaP-C42 cell adhesion to ICAM-1 and fibrinogen (Figure 5B and C), thus demonstrating that simvastatin suppressed interactions between prostate cancer cell-surface integrin $\alpha_v\beta_3$ and ICAM-1 that are abundantly expressed by endothelial cells.

Simvastatin enhances endothelial cell-barrier integrity via βcatenin stabilization

Next, we determined the direct effects of simvastatin on vascular endothelial-barrier junctions. To investigate this, we performed immunocytochemistry analysis of endothelial monolayer subjected for dose- and time-dependent effects of simvastatin, with antibodies

specific for βcatenin. βcatenin expression analysis of HMVECs treated with simvastatin for 6 h indicated a significantly elevated βcatenin expression in HMVEC-barrier junctions with 5 μM simvastatin with modest, but significant reduction in expression βcatenin with 25 μM simvastatin (Figure 6 A and B). These results indicated that simvastatin elicits vascular protective effects via endothelial-barrier enhancement. However, our results also provide the need for caution at the use of very high doses of simvastatin for prostate cancer treatment as higher doses can be toxic to endothelial cells and normal vasculature.

Discussion

The process of micrometastasis involving intravasation and extravasation of cancer cells is an essential pre-requisite for the cancer cells to metastasize to distant tissues (Steffen et al., 1994). Our current study demonstrates the potential benefits of simvastatin in the inhibition of prostate cancer metastasis (Figure 7). We first showed that simvastatin treatment inhibited transendothelial migration of highly invasive PC3 cells in an ECIS array equipment *in vitro*. Our gene array experiment, followed by Western analysis identified a decrease in VEGF-A expression and increase in Angiopietin-1 expression by PC3 cells in response to simvastatin treatment. WOW-1 binding assay that measures the surface expression of activated integrin $\alpha_v\beta_3$ in PC3 cells and fibrinogen binding assay demonstrated the effect of simvastatin on significantly inhibiting the affinity of integrin $\alpha_v\beta_3$ to bind to its ligands. Co-treatment with AP7.4, integrin $\alpha_v\beta_3$ activating antibodies, rescued simvastatin-mediated inhibition of PC3 cell micrometastasis. While pre-treatment with simvastatin significantly impaired the ability of PC3 cells to bind to the endothelial cell surface, adhesion of PC3 cells to soluble ICAM-1, adhesion molecules that are abundantly expressed in endothelial cells (Dustin et al., 1986) often implicated in mediating transendothelial migration of inflammatory cells (Steiner et al., 2010) and cancer cells (Conrad et al., 2009) was significantly blunted by treatment with simvastatin. Finally, simvastatin treatment stabilized the vascular adherens junctions and protected endoethelial-barrier by enhancing β-catenin expression. Collectively, our data demonstrates the potential therapeutic benefits of simvastatin in preventing prostate cancer micrometastasis.

Much of the effects of simvastatin on prostate cancer micrometastasis have been attributed to its ability to differentially modulate cell signaling in normal cells at rest and malignant cells. While statins have been previously shown to inhibit proliferation of vascular smooth muscle cells in atherosclerotic plaques (Liao, 2005) and inhibition of cell motility and migration of various cancer cells (Goc et al., 2012d), including prostate cancer cells (Goc et al., 2011, Goc et al., 2012b), vascular endothelium is protected by statins via activation of Akt-eNOS pathway (Kureishi et al., 2000). Hence, while statins protect the vasculature, it inhibits tumor growth and may prevent micrometastasis. This ability of statins to normalize the tumor cells and the cells in the microenvironment provides the benefit of improving the efficacy of chemotherapeutic drugs when treated in combinations with statins.

Our previous results related to the effects of simvastatin on prostate cancer cells *in vitro* and tumor xenograft growth *in vivo* are completely in agreement with the general perception that statins normalize the deregulated signaling pathways in malignant cells. We showed that while simvastatin inhibited prostate cancer cell proliferation, migration and colony

formation *in vitro*, it impaired the growth of tumor xenografts *in vivo* (Kochuparambil et al., 2011). In addition, simvastatin induced apoptosis in prostate cancer cells via inhibition of intrinsic cell survival as well as activation of extrinsic death-receptor pathways (Goc et al., 2012b). Results from the current study on the specific effects of simvastatin on micrometastasis of prostate cancer cells *in vitro* further strengthens our overall idea of utilizing statins for prostate cancer therapy either alone (for prevention) or in combination with chemotherapeutic drugs such as Taxotere®(for therapy). Our studies suggest that dual effects of simvastatin on inhibition of interactions between the prostate cancer cells and the endothelium, and on the modulation of tumor-derived factors such as VEGF and angiopoietins will provide a net stabilizing effect on the tumor vasculature, by reducing the vascular permeability, an important feature of tumor vasculature (Goel et al., 2011).

A very recent study has also demonstrated the effect of atorvastatin on VEGF-A expression by the human small non-cell lung carcinoma cell lines (Chen et al., 2012). While our study confirms this report to be true also in the case of prostate cancer cells, we provide additional information on the inhibitory effect of simvastatin on the expression of angiopoietin-2, a signaling companion of VEGF in the modulation of tumor vascular permeability (Gale et al., 2002), and enhance the expression of angiopoietin-1, a growth factor that stabilizes the vascular endothelial-barrier junctions, thus stabilizing the endothelial-barrier and reducing vascular permeability (Gale et al., 2002). Other prominent pro-angiogeneic growth factors or signaling molecules identified in the gene array that are regulated by simvastatin in PC3 cells include TEK-Receptor tyrosine kinase, Insulin like growth factor-1 (IGF-1), integrins α_4, β_3 and β_5 and FGF Receptor-2, all of which are inhibited by simvastatin. Since IGF-1 has been implicated in the development of castration resistant prostate cancer (Koutsilieris et al., 2000) and integrins are involved in micrometastasis, simvastatin treatment appears to be an effective treatment strategy to prevent recurrence after prostatectomy and prevent further spread as has been shown recently (Hamilton et al., 2010). Furthermore, staining of endothelial-barrier junctions for βcatenin, a predominant adherens junction protein indicated enhanced endothelial-barrier protection upon treatment with simvastatin, accompanied by enhanced barrier-resistance. Interestingly, these changes in gene and protein expression of angiogenic modulators by simvastatin did not have a net effect on tumor angiogenesis *in vivo*, indicating that simvastatin provides a normalizing and stabilizing effect on the tumor endothelium. This priming effect on the tumor vasculature is a feature considered essential for anti-angiogenic therapy (Al-Husein et al., 2012).

Impaired micrometastasis of PC3 cells pre-treated with simvastatin in an ECIS array suggested that factors other than tumor derived factors may be involved in the modulation of simvastatin-mediated prostate cancer micrometastasis. An important mechanism by which circulating blood cells and cancer cells transmigrate the endothelial-barrier is via a direct heterophilic interaction with the endothelium mediated through cell-surface integrins and cellular adhesion molecules (Steiner et al., 2010, Conrad et al., 2009). Statins have previously shown to impair transendothelial migration of inflammatory cells via inhibition of their interaction with the endothelium (Steiner et al., 2010). Our previous studies have shown that Akt and Rac pathways enhance prostate cancer micrometastasis involving integrin $\alpha_v\beta_3$ (Goc et al., 2012a, Goc et al., 2012d), which are extracellular matrix (ECM)

receptors abundantly expressed in prostate cancer cells and have been implicated to be necessary for prostate cancer metastasis (McCabe et al., 2007). Since Akt and RhoGTPases are important targets of simvastatin in cancer cells (Kochuparambil et al., 2011, Ghosh et al., 1999) we postulated that simvastatin-mediated inhibition of PC3 micrometastasis may involve integrin $\alpha_v\beta_3$. Our findings supported this hypothesis and demonstrated that while simvastatin treatment resulted in impaired inside-out activation of integrin $\alpha_v\beta_3$ and thus reduced their affinity for specific ligands such as fibrinogen. Furthermore, co-treatment of PC3 cells with simvastatin and AP7.4 (integrin $\alpha_v\beta_3$ activating antibodies) rescued the impaired micrometastasis, once again confirming that simvastatin has a direct effect on integrin $\alpha_v\beta_3$ on reducing its ligand affinity.

Cell-surface integrins on circulating blood cells and cancer cells often interact with cell adhesion molecules expressed on endothelial cells such as VCAMs and ICAMs (Steiner et al., 2010). Among these, interaction between integrin $\alpha_v\beta_3$ and ICAM-1 is the best characterized (Conrad et al., 2009). Our data indicated that pre-treatment with simvastatin significantly impaired the ability of PC3 cells to recognize and adhere to human soluble ICAM-1, thus demonstrating the direct effect of simvastatin on inhibiting interactions of prostate cancer cells with cellular adhesion molecules. Although not tested in the current study, statins have been previously reported to reduce the expression of cellular adhesion molecules such as ICAMs and VCAMs on endothelial cell surface (Dustin et al., 1986). Hence, simvastatin is expected to elicit a much more potent inhibitory effect on prostate cancer micrometastasis *in vivo* as compared to our unidirectional approach in the *in vitro* experiments. Altogether, this study identifies simvastatin as a potent inhibitor of prostate cancer micrometastasis via inhibition of tumor derived factor expression, inhibition of prostate cancer cell transendothelial migration and by stabilizing the endothelial-barrier. These results combined with the anti-inflammatory and endothelial-barrier stabilizing effects of simvastatin suggest that statins could be re-purposed for the management of prostate cancer either alone or in combination with chemotherapeutic regimen.

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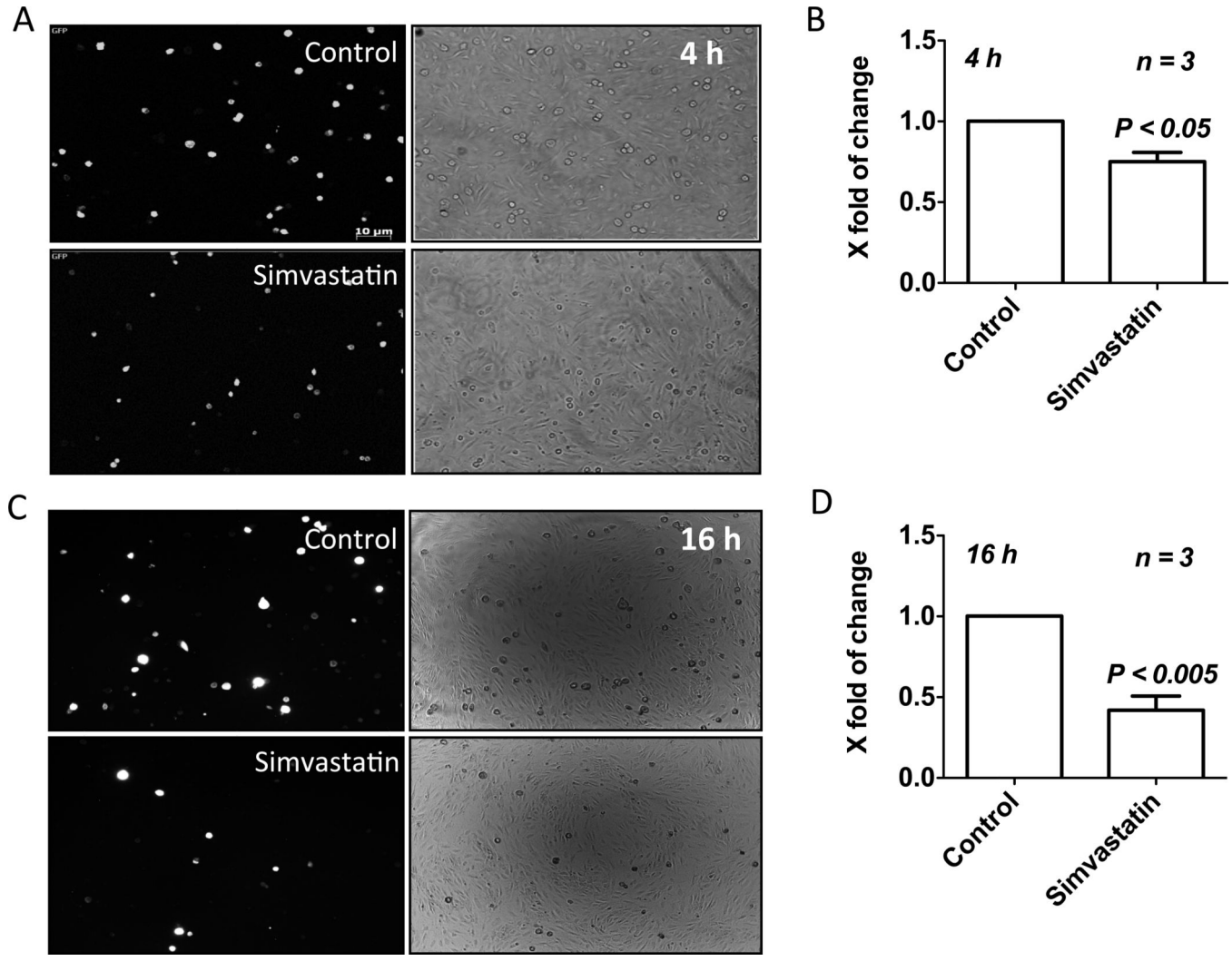

Figure 1.

Simvastatin inhibits binding of PC3 cells to the endothelial monolayer. (A–D) PC3 cells expressing GFP were treated with 25 μM simvastatin for 4 h (A and B) and 12 h (C and D), and introduced into 12-well plates containing a confluent monolayer of HMVECs. One hour later, medium was removed, wells were washed with PBS and the number of GFP-positive PC3 cells were determined.

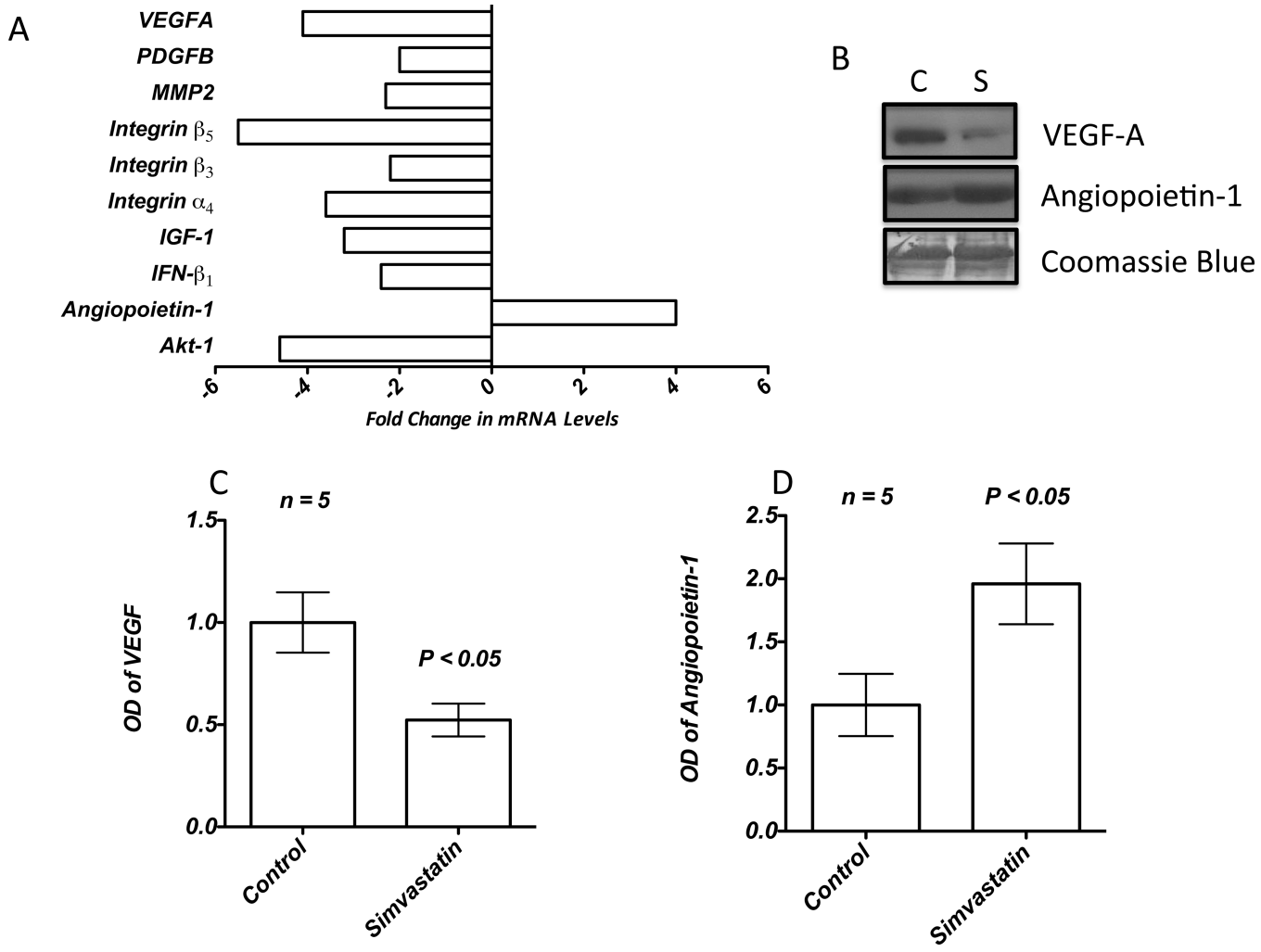

Figure 2.

Simvastatin modulates expression of secreted angiogenic factors by the PC3 cells. (A) Bar graph showing qRT-PCR array data indicating key genes modulated by simvastatin. (B) Western analysis of PC3 cell conditioned medium concentrated by TCA-precipitation showing changes in the expression of VEGF-A and Ang-1. (C and D) Band-densitometry analysis of the Western blots of PC3 cell conditioned medium indicating changes in the expression of VEGF-A and Ang-1 normalized to coomassie staining.

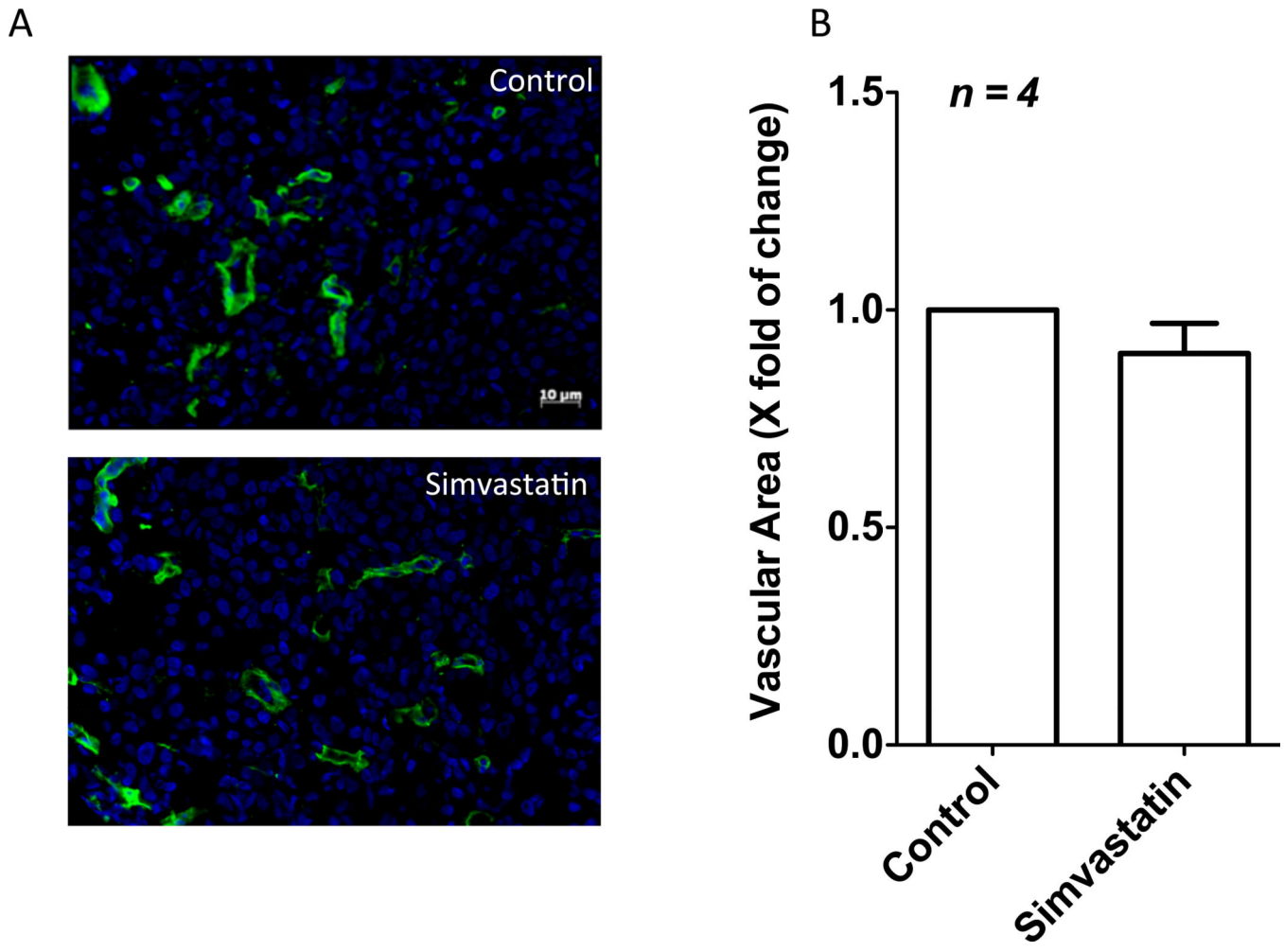

Figure 3.

Simvastatin has no effect on prostate tumor angiogenesis. (A) Immunofluorescence staining of PC3 tumor xenograft sections from nude mice showing laminin staining as a measure of tumor angiogenesis. (B) Bar graph showing no differences in the vascular area in tumor xenografts between c ontrol (saline) and simvastatin (2mg/kg/12 h) treated mice.

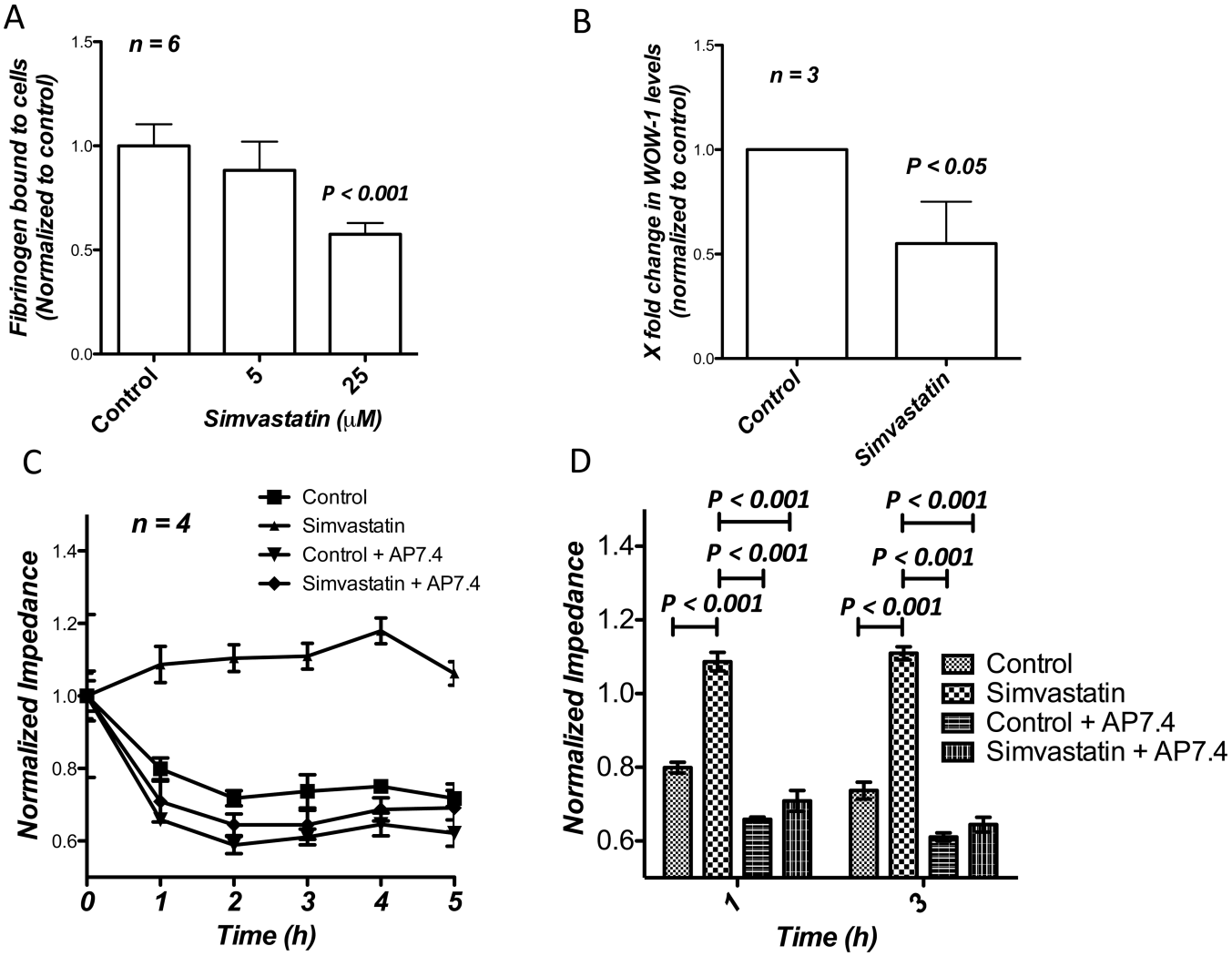

Figure 4.

Simvastatin abolishes PC3 cell interaction with the endothelium via inhibition of PC3 cell surface integrin $\alpha_v\beta_3$ affinity for its ligands. (A) Bar graph showing the effect of simvastatin on PC3 cell interactions with Alexa486-labelled fibrinogen, an integrin $\alpha_v\beta_3$ ligand. (B) Bar graph showing impaired binding of WOW-1, a specific ligand for activated integrin $\alpha_v\beta_3$, by the PC3 cells treated with 25 μM simvastatin compared to control. (C and D) Figure showing significant inhibition of PC3 cell transendothelial migration with 25 μM simvastatin compared to control saline treated cells. Co-treatment with AP7.4, integrin $\alpha_v\beta_3$ activating antibodies abolished simvastatin-mediated effects.

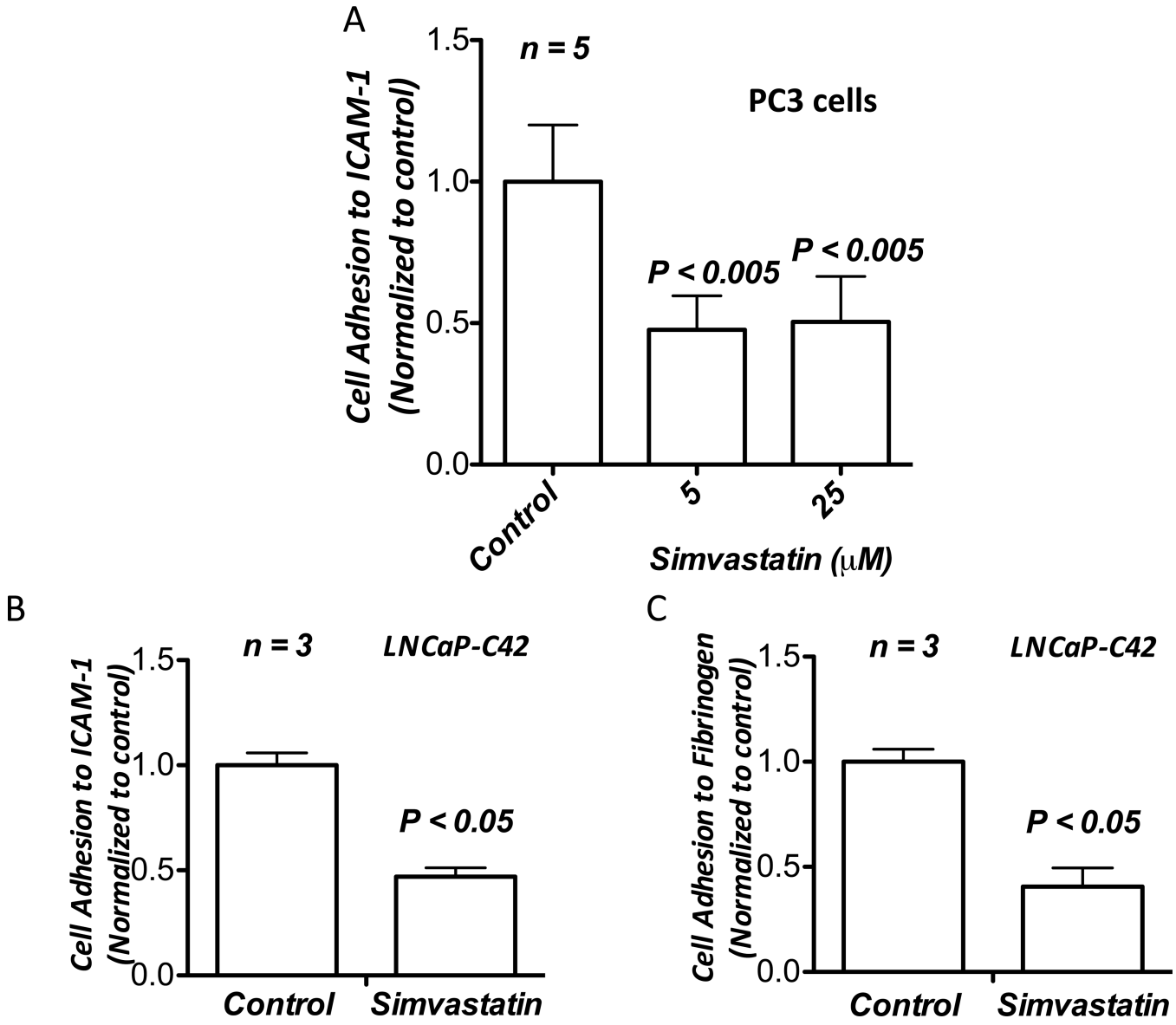

Figure 5.

Simvastatin inhibits interaction between prostate cancer cell-surface integrin $\alpha_v\beta_3$ and endothelial ICAM-1. (A) Bar graph showing significant inhibition of adhesion of PC3 cells on pre-coated soluble human ICAM-1 by treatment with 5 and 25 μM doses of simvastatin. (B) Bar graph showing inhibition of adhesion of LNCaP-C42 cells on pre-coated soluble human ICAM-1 by treatment with 25 μM simvastatin. (C) Bar graph showing inhibition of adhesion of LNCaP-C42 cells on pre-coated fibrinogen by treatment with 25 μM simvastatin.

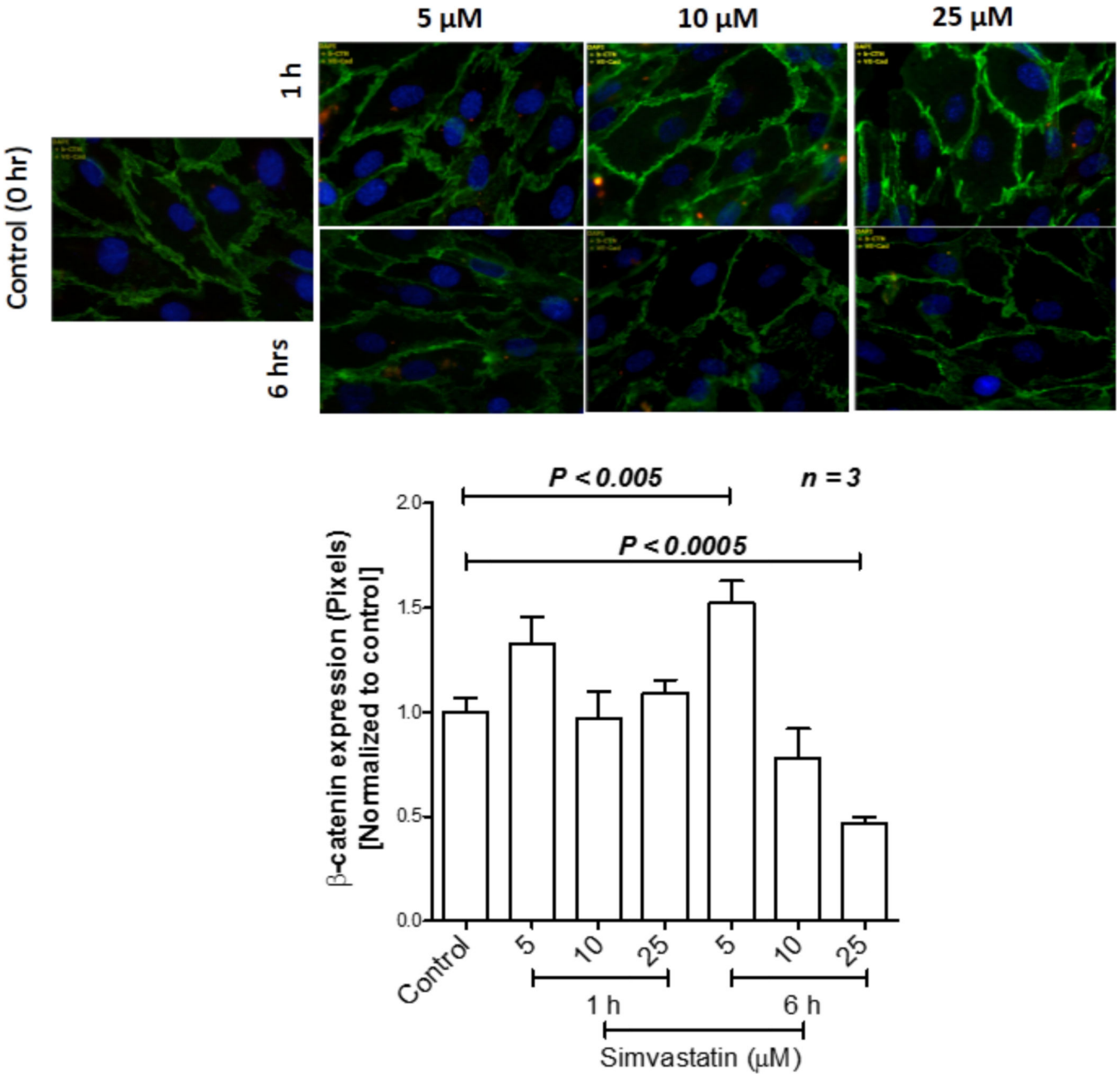

Figure 6.

Simvastatin stabilizes the endothelial-barrier in mediating normalization of the tumor vasculature. (A) Fluorescent microscopic images of HMVEC monolayer stained with adherens junction protein β-catenin. (B) Bar graph of the above data indicating changes in expression with 5, 10 and 25 μM doses of simvastatin.

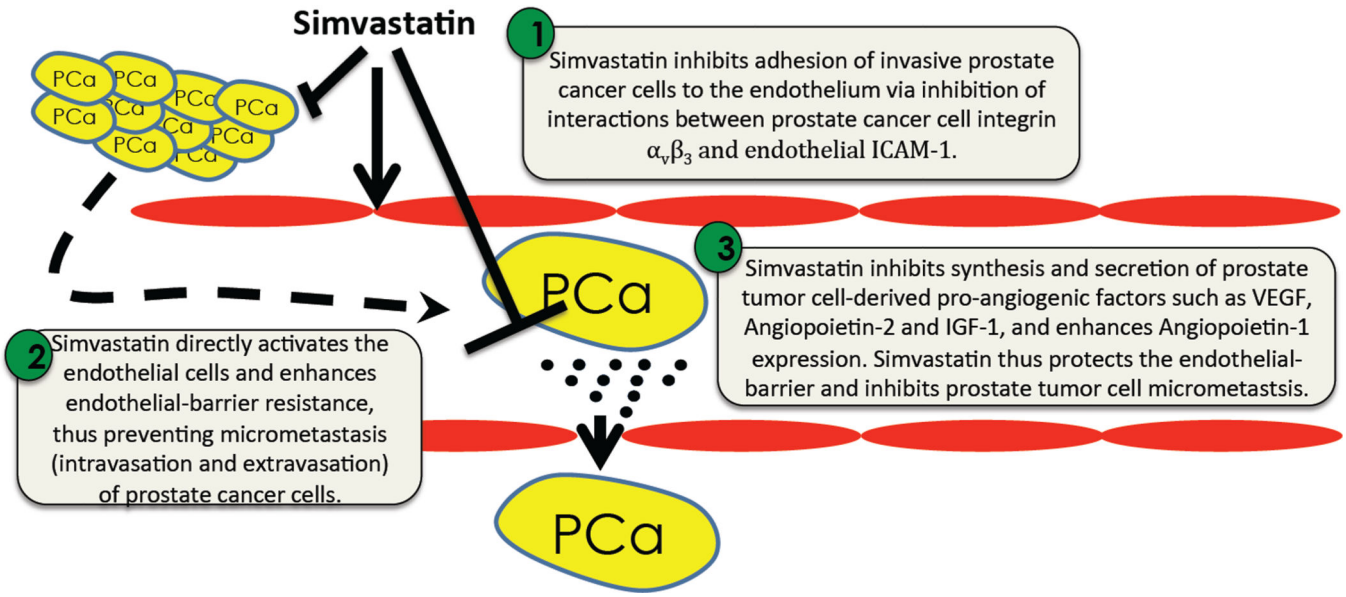

Figure 7.
Schematic representation of the working hypothesis.

Table 1

Key angiogenic genes modulated by simvastatin in prostate cancer (PC3) cells

Gene Symbol	Gene description	Change (X-fold)
AKT1	V-akt murine thymoma viral oncogene homolog 1	4.6↓
ANGPT1	Angiopoietin 1	4.0↑
ANGPT2	Angiopoietin 2	1.8↓
CHEK2	CHK2 checkpoint homolog	1.6↓
COL18A1	Collagen, type XVIII, alpha 1	4.7↓
E2F1	E2F transcription factor 1	8.2↓
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2	6.3↓
FGFR2	Fibroblast growth factor receptor 2	1.8↓
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog	1.6↓
IFNB1	Interferon, beta 1, fibroblast	2.4↓
IGF1	Insulin-like growth factor 1 (somatomedin C)	3.2↓
ITGA4	Integrin, alpha 4 (CD49D)	3.6↓
ITGB3	Integrin, beta 3 (glycoprotein IIIa, CD61)	2.2↓
ITGB5	Integrin, beta 5	5.5↓
JUN	Jun oncogene	5.1↓
MMP2	Matrix metallopeptidase 2	2.3↓
MTA2	Metastasis associated 1 family, member 2	3.8↓
MTSS1	Metastasis suppressor 1	2.0↓
MYC	V-myc myelocytomatosis viral oncogene homolog	3.7↓
PDGFB	Platelet-derived growth factor-β	2.0↓
PLAUR	Plasminogen activator, urokinase receptor	1.8↓
PNN	Pinin, desmosome associated protein	1.5↓
SNCG	Synuclein, gamma	3.1↓
SYK	Spleen tyrosine kinase	2.0↓
TEK	TEK tyrosine kinase, endothelial	1.9↓
VEGFA	Vascular endothelial growth factor A	4.1↓